

The plight of the dismal science

Economists in the United States are offended at their representation on the new president's economic councils, but there is a sense in which they can blame only themselves.

PRESIDENT Bill Clinton had offended many professional economists even before he formally succeeded President George Bush this week. The dismal science is offended not by what little can be gleaned from past speechmaking about US economic policy in the next few years, but by Clinton's appointments to his Council of Economic Advisors. That is the administration's chief source of advice on economic issues; it has a role in Washington comparable with that of the Office of Science and Technology Policy, but commands more immediate attention because economic issues cannot as easily be dodged. What academic economists are complaining about is that Clinton's council includes nobody of outstanding stature among themselves. When the Nobel Prize in economics has become virtually a US monopoly, they feel slighted that Clinton has appointed lesser mortals to the council.

Economists should not be so surprised. Although there are grounds for worrying that the new council will be soft in its judgement of proposals for government intervention in industry, or for the management of 'free' trade, Clinton could easily have recruited academics of the highest reputation and with the same inclinations. If slight there is, it may just as well be read as the impatience of the ex-governor of one of the poorest states in the United States with the common (but not universal) tenor of academic economists' views on current economic problems. The trouble is not, as some say, that economics has become 'too mathematical', but that contemporary economics pays too little regard to the limitations of the models of economic behaviour it constructs.

That is not dismissive of economists, but rather an acknowledgement of the difficulties they face. Models of national economies, all the rage in the past 20 years, readily illustrate the point. The British Treasury, having laboured through the 1970s on a model of the British economy, was then proud enough of its achievement to make the model generally available. On the face of things, the Treasury had stolen a march on the weather forecasters, still then struggling to build reliable computer models of the atmosphere. But the advance has always been an illusion, as the frailty of the British Treasury's economic forecasts now amply demonstrates. Yet, oddly, economic modellers themselves are shy of acknowledging that theirs is a much more complicated (or technically 'complex') problem than even the weather forecasters have had to tackle.

Meteorologists continually complain about the adequacy of the data by which they calibrate their models, but economists must work with even more flimsy datasets. Weather observations are now available almost in real time, but economists must make do with aggregate measurements of, say, industrial output or exports that are aggregated once a month, are subject to horrendous statistical noise (occasioned by, for example, the delivery of aircraft from a manufacturer to a purchaser) and which are then 'revised' as succeeding months pass.

More seriously, while meteorological modellers can be confident of the physical basis of their models, economists are embedded in shifting sand. Instead of Newton's laws, they often have to guess at human reactions to changed economic circumstances. Plainly, from the experience of the past three years, they have seriously underestimated the degree to which personal indebtedness and the fear ▶

Meetings on British science

NATURE is organizing two one-day public meetings in Edinburgh and London to discuss proposals for the forthcoming white paper on the organization of British science.

On Friday 19 February, there will be a meeting in **Edinburgh**, jointly sponsored by the Edinburgh International Science Festival. The speakers will be Professor John Glover (University of Dundee), Sir Graham Hills (formerly of the University of Strathclyde), Professor J Maxwell Irvine (University of Aberdeen) and Professor David Wallace (University of Edinburgh). The meeting will be at the Queen Mother Conference Centre, Royal College of Physicians, 9 Queen Street, Edinburgh, and will start at 9.30 a.m. Admission will be by ticket, free of charge, obtainable from Bruce Durie, Edinburgh International Science Festival, 1 Broughton Market, Edinburgh EN3 6NU.

On Friday 19 March, there will be a meeting in **London** at the Royal Society, 6 Carlton House Terrace, SW1. The speakers will include Sir Mark Richmond (Chairman, Science and Engineering Research Council) and Professor Michael Brady (University of Oxford), and the meeting will start at 9.30 a.m. The other speakers will be announced later. Admission will be by ticket, free of charge, obtainable from Mary Sheehan, *Nature*, 4 Little Essex Street, London WC2R 3LF. □



of unemployment have prevented people from spending what incomes they are left with, thus prolonging the current recession. Economists in general cannot be blamed for that failure — which nevertheless suggests that social science inquiries should be more durably part of economics — but only those who have been led by fondness for their models to promise too much for them.

Much the same is true of other current arguments. Is monetarism (the doctrine that economies can be regulated by controlling the supply of money) still a valid recipe, for example? Whatever the truth, the experience of the past few years shows that governments faced with recession prefer Keynesian recipes; employment becomes more important than anti-inflationary strategies. So forecasters must guess at the behaviour of governments as well as people. Clinton's choice of economic advisers may thus have been determined by a wish not to be impeded in what he knows he wants to do by academic debate about the theoretical basis of his policies. The economists who feel slighted should shake off the sense of slur, and regard this development as a challenge instead. □

Patent for a mouse

Europe is making heavy weather of the patenting of transgenic animals and plants.

SHOULD strains of animals and plants be patentable? Even when, or because, they are made by genetic engineering? This issue seems destined to become another issue with which the biotechnology industry will be harassed for many years to come. On the heels of the granting of a patent for what is called the Harvard oncomouse, there was a gathering of pressure groups at Munich earlier this month (see *Nature* **361**, 103; 1993) promising appeals against the patent and further protests if patents are granted to those who have developed other transgenic animals now being considered by the European Patent Office (EPO). It is significant that EPO's rules differ from those of the US Patent Office, which granted a patent for the mouse in 1988.

The present fuss follows EPO's decision first refusal of Harvard's patent application and a successful appeal against it. The mouse itself is not so much a breeding stock of animals as a concept (see Stewart, T.A., Pattengale, P.A. & Leder, P., *Cell* **38**, 627–637; 1984). By linking the gene for the *myc* oncogene with altered promotor regions and incorporating the altered genes in animals, the designers found they had made animals that develop breast tumours readily during pregnancy or under endogenous influences. Among other things, the mice are potential models of carcinogenesis in general and also vehicles for the testing of both drugs and oncogenic influences.

This Harvard mouse is hardly typical of those waiting in the queue for patents. It is a research tool only, strictly comparable with the mice of known strain that specialized laboratory suppliers put on sale. Quite why Harvard Uni-

versity thought it worthwhile applying for a patent is not self-evident, although the arrangement will make possible the orderly supply of the transgenic animals when researchers do not prefer to make their own. Yet the sales are unlikely to be huge, while there will be many researchers who prefer to make their own. But in principle there can surely be no objection that the designers of the oncomouse should derive some benefit from the genome they have constructed, and that they or their institution should be rewarded.

The reasons why EPO first rejected the application on behalf of the Harvard mouse are nevertheless significant. EPO was swayed by the clause in its statute requiring that inventions should not be patented when their use would be "contrary to public order or morality". (Taylor Joynson Garrett, the London lawyers who also act for *Nature*, remark in their periodical *Intellectual Property Review* that this clause has been loosely translated into British law as "generally expected to encourage immoral, offensive or anti-social behaviour".) The pressure groups at Munich were arguing that the laboratory use of animals entailed and implied by the applications for a patent is immoral.

The difficulty for EPO is that both the construction and the use of the Harvard mouse would be allowed under European law (under appropriate licence) and are thus presumably moral. It will be a nonsense if EPO remains saddled with an injunction to issue patents only when it has made a separate and supranational decision about the proposed use of transgenic animals and plants, becoming in the process a second European Court. EPO would be well rid of that responsibility.

More taxing issues relate to patent applications for transgenic animals and plants intended for use in agriculture. Uses of that kind are strictly analogous to the present use of varieties of plants or pedigrees of animals, which are covered not by patent law but by registration schemes operated by most governments. Breeders earn their rewards by charging extra for seed or semen (but stud fees are an equivalent). Farmers may then make what use they choose of the genes they have acquired, provided they do not sell them on the commercial market, while other breeders are free to emulate pioneers in the field.

There will be an awkward muddle if patented plants and animals must now coexist with those covered by registration schemes. Will the patent-holders seek a huge capital sum for the first supply of novel genetic material, or alternatively seek a royalty from future crops or animals descending from the original stock supplied? Moreover, the techniques of genetic grafting are now moving so quickly that today's wonders will seem unremarkable a few years from now. Will it then seem fair that genetic engineers other than the patent-holder should be prevented from developing, say, a chicken with extra growth hormone genes? That potentially damaging muddle is a more serious threat to the EPO's than the morality clause it is now saddled with. □

Six volcanologists killed in Colombian eruption

London. In an ironic twist of fate, six scientists are believed to have died in a volcanic eruption in Colombia last week as they worked on a monitoring system to help predict future eruptions and thus save lives. Among those killed on the 14,000-foot Galeras volcano was Geoffrey Brown, professor of Earth sciences at Britain's Open University and a pioneer in the use of gravity measurements to help increase the accuracy of predictions about when and where a volcano is likely to erupt.

Galeras has historically been the most

two eruptions occurred about two hours apart. The first, which trapped those in the crater, lasted about 15 minutes.

Among those injured by the eruption was the main organizer of the workshop, Stan Williams of Arizona State University, who has been studying the volcano for several years. He is said to have been pulled to safety from 30 metres down in the crater by a Colombian geologist from the National Institute for Geological and Mineral Investigations and a driver on the expedition. Williams was subsequently flown back to the United States for medical treatment.

Other scientists who had moved further into the crater, including Brown, were all killed. At the time of the eruption, several of them were installing equipment to monitor gases escaping from the dome.

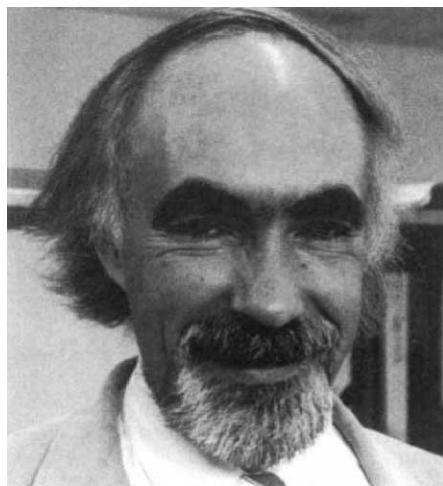
Brown was setting up equipment to measure the gravitational field in the dome. Small changes can result from shifts in the magma within a volcano. In previous studies in Costa Rica and Europe, Brown and his colleagues have shown that such changes may provide clues to eruptions not provided by seismic data or measurements of surface distortion. "It was a type of reconnaissance exercise to see if we could set up a gravity monitoring network", says Hazel Rymer, who has worked closely with Brown in developing the techniques.

Although information is still scanty, a spokesman for the British Foreign Office said earlier this week that nine bodies had been recovered and that three more people were still unaccounted for. The dead scientists were mostly from Colombia, but also included an unidentified researcher from the Institute of Volcanology in Russia.

This week, colleagues at the Open Uni-



The Galeras volcano is near the border with Ecuador in southwestern Colombia.



Eruption killed Geoffrey Brown.

active volcano in Colombia, erupting 21 times since 1580. It has been under close observation by scientists since first indications of renewed activity in 1988. A small eruption in May 1989 was followed by another in October 1991, and then by a more violent explosion last July, which destroyed the lava dome.

Such precursors of a major eruption (a tilting of the dome was observed in November), together with the fact that it is located only 8 km from the centre of the town of Pasto, made Galeras one of seven volcanoes singled out for special attention under the United Nations International Decade of Natural Disaster Reduction.

The scientists who died were taking part in a workshop organized by Colombian, Canadian and US scientists. One of the main goals of the workshop was to develop a strategic plan for monitoring the volcano's present activity and interpreting its geological past.

Part of the workshop involved a day-long field trip on 14 January to the active crater to collect gas samples and make other observations. According to reports received from Colombia, it was during this visit that

versity paid tribute both to Brown's work for the Earth sciences department and to his activity as head of the university's research committee. In the latter position, he had been responsible for coordinating the university's participation in the recent research evaluation exercise carried out by the Universities Funding Council.

"Geoff passionately believed that good teaching should be supported by good research", said Chris Wilson, acting head of the department. "His role was crucial in the research selectivity exercise, and also in the way that research in the department has flourished."

Early next month, the department is opening a new laboratory wing, described by Wilson as a "crowning achievement" to Brown's work for the university. The building has been partly funded by the Wolfson Foundation and will be the first specifically built for research purposes on the Open University campus.

David Dickson

EPA makes small field tests easier

Washington. In an attempt to clear the decks before the new administration takes office, the US Environmental Protection Agency (EPA) last week issued its long-awaited rule on small-scale field testing of microbial pesticides.

Under the new rule, EPA, which regulates the use and distribution of pesticides under the Federal Insecticide, Fungicide and Rodenticide Act, will require prior notification of small-scale field testing in the environment for only a small subset of microbial pesticides — those posing the greatest potential risks or where there is insufficient information about the potential risk of

introduction into the environment. Notification is now required for all genetically altered and nonindigenous microbial pesticides. The new rule applies only to small-scale field tests. Companies carrying out field tests of more than 10 acres will still be required to apply routinely for experimental use permits from EPA.

The new proposal was welcomed by the Industrial Biotechnology Association, which has worked closely with the Bush administration for more than two years to clarify the scope of the agency's supervision and reduce the regulatory burden on the industry.

Diane Gershon

Historic Bart's fights for its life under medical school reorganization

London. The British government is having second thoughts about a plan to close St Bartholomew's Hospital Medical School in London that has generated more than four hundred letters of protest from scientists around the world.

Teaching and research at the school — affectionately known as Bart's — would be transferred to the Royal London Hospital Medical College in East London under proposals submitted to the government for the rationalization of London's health services by a committee headed by Sir Bernard Tomlinson. The merger would require Bart's to sell its current buildings, including its Great Hall adjacent to the old Smithfield meat market.

A spokesman for the Royal London, with which Bart's has been linked in a relatively loose federation for several years, said last week that a full-scale merger would be welcomed. "We believe that by joining with Bart's we would have a greater critical mass than we have now, and that can only be good for research."

However, after a meeting last week with John Major, the prime minister, the secretary of state for health, Virginia Bottomley, was widely reported as planning to hold back from immediate implementation of the more radical proposals in the Tomlinson report. In particular, she is expected to endorse an evolutionary change that might incorporate Bart's own counter-proposal that it should withdraw from treating general patients and concentrate on special conditions, such as cancer and AIDS.

Hostility to the merger with the Royal London remains high at Bart's — the hospital was founded in 1123 and the medical school in 1662 — and is based primarily on concerns about maintaining the close relationship between clinical and basic research, as well as between different research disciplines, which the medical college has built up in recent years. "This is an environment which provides the right mix of disciplines for me, and where I can do what I want to do", says AIDS researcher Tony Pinching; "You can't transplant an ethos."

The Tomlinson report reflects two related changes being pushed by the Conservative government. The first is in the organization of National Health Service hospitals. Based on the concept of an 'internal market', the hospitals are now required to contribute directly to the costs of specialist services; constraints in funding for this in regional hospitals have reduced the number of patients attending specialist services in London, and thus the availability of research subjects (see *Nature* 357, 617; 1992). The second change has been to encourage

universities to rationalize their research funding, concentrating on building up centres of scientific excellence and closing down or merging weaker research departments.

Combining these principles, Tomlinson recommended that all but one of London's nine undergraduate medical schools be merged into four faculties of medicine within the multi-faculty colleges of the University



Will Bart's doors stay open?

of London. Undergraduate clinical teaching would be increasingly dispersed to hospitals outside the centre of the city. In contrast, clinical laboratories would be concentrated in a limited number of inner-city teaching hospitals, these being "as far as possible close to a basic science base".

Not surprisingly, medical schools that stand to benefit from the changes have reacted favourably to the report. For example, researchers at University College Medical School — which recently linked up with Middlesex Hospital — have welcomed the suggestion to strengthen the connection and to proceed with the joint construction of a new teaching and research block.

A more cautious response has come from the medical profession. The British Medical Association has emphasized that, if there is to be a rationalization of medical schools, "it

is essential that successful research teams should not be split up but should be relocated en masse". Similarly, a working party representing the medical royal colleges, which oversee the training of doctors, says that the necessary closure and rationalization of hospitals and units must be planned in a way that protects the research.

The likely losers from a radical reorganization have been mounting a campaign to persuade the government to modify Tomlinson's proposals. Last Friday, for example, a petition containing half a million signatures was delivered to 10 Downing Street by staff from the Royal Marsden Hospital (which specializes in cancer treatment) and the Royal Brompton National Heart and Lung Hospital. The report recommends a merger of both with the Charing Cross Hospital, a step the institutions see as potentially damaging to their scientific reputations. A similar number of signatures has been collected in defence of Bart's.

One important factor in the college's favour is the poor state of the London property market, and the fact that, because most of its buildings are historic monuments, commercial development would inevitably be hampered by planning restrictions.

The Tomlinson report suggests that the cost of rationalization should be financed primarily from the sale of the assets of medical schools. In particular, it valued the Bart's site and buildings at about £160 million. At the college itself, however, this is felt to be a wild overestimate; its own evaluation suggests a value of £18 million or less. A study commissioned by the University of London concludes that implementing the proposals could cost the university between £74 and £135 million.

Although relatively small in terms of what is spent by the health service, this would represent a major expenditure for the higher education system. The government's response, expected in the middle of next month from Bottomley, will be determined in large measure by whether it is prepared to meet this sum.

Meanwhile at Bart's, they are not dropping their guard, and point out that rumours of a reprieve are speculative. "This place has a spirit that we have a responsibility to defend", says Lesley Rees, professor of endocrinology and dean of the college.

David Dickson

Correction

Regrettably, the map accompanying our supplement on Nordic science (see *Nature* 360, 509–528; 1992), which was intended to remind readers of the geography of Scandinavia, inadvertently omitted Poland. *Nature* apologizes for this omission.

US military accommodates astronomers

Washington. Astronomers have persuaded the US Pentagon's Strategic Defense Initiative (SDI) to change its plan to orbit a nuclear reactor that might have interfered with observations by astronomy satellites. Managers of the SDI project to orbit the reactor as a test of nuclear propulsion in space have agreed to do whatever is necessary to avoid compromising the scientific return from the satellites, even if it means paying for a more expensive rocket to lift the Topaz 2 reactor into a higher orbit.

Astronomers first became concerned about the Topaz mission last September, when they learned that the SDI office intended to place the Russian-built reactor into an orbit with an altitude of 1,600 km. Similar reactors flown by the Soviets at lower altitudes in the 1980s had interfered with two high-energy astronomy missions: Japan's Ginga X-ray satellite and the US Solar Maximum Mission satellite. The interference comes from two types of reactor emissions: fission gamma rays, which cloud the cosmic X-ray background, and secondary particles, which can give false readings when they strike sensitive detectors.

A launch is planned for 1995, when as many as ten satellites observing in X-ray and gamma-ray wavelengths will be either in space or on the way to the launch pad. The mission most in jeopardy would be the National Aeronautics and Space Administration (NASA)'s \$617-million Compton Gamma Ray Observatory, launched in 1991. According to calculations done at the Naval Research Laboratory, the Compton satellite

would get as many as 30 false readings a day if Topaz were placed in an initial orbit of 1,600 km, and one of its instruments for detecting gamma-ray bursts would effectively be blinded.

Astronomers approached SDI with their concerns, and in October a NASA advisory panel requested that the reactor be placed in an orbit above 6,000 km. At that distance, the Compton observatory would record one false event a day, thought to be a manageable level. But in December, after SDI appeared ready to solicit bids for a launch vehicle that could reach only a nominal 1,600-km orbit, the astronomers launched their own media campaign.

That effort, led by Don Lamb of the University of Chicago, who chairs the high-energy astrophysics division within the American Astronomical Society (AAS), culminated in a resolution passed by the society at its annual meeting on 11 January, urging SDI to place the reactor in a higher orbit. A press release accompanying the resolution, timed to coincide with SDI's deadline for receiving launch bids for Topaz, led to coverage in the media. Two days later, managers of the \$150-million project were assuring scientists that the reactor would not be launched until it had met their requirements.

Both sides have remained civil throughout the controversy, commending the other side's willingness to search for a reasonable solution. The SDI office says that it was never committed to the 1,600-km altitude, that non-interference with scientific missions is important and that there are some advantages to a higher orbit anyway. For their part, the astronomers say that it was all just a misunderstanding. "I think they [SDI officials] didn't realize how many satellites this would affect", says Peter Boyce, AAS's executive officer.

It is not known if a 6,000-km altitude will solve the problem. More work is needed on predicting the effects of Topaz on astronomy satellites, an issue due to have been taken up earlier this week at a meeting involving US and Russian scientists at the University of Maryland. SDI believes that extra shielding may allow the reactor to be placed in a lower orbit without causing undue interference.

Meanwhile, both sides seem happy. The Pentagon can focus on what are expected to be more vociferous protests from anti-nuclear groups arising nearer to the launching of Topaz, while astronomers are pleased to have gotten their way. "It's the way you would like to be able to resolve problems", says Boyce.

Tony Reichhardt

Healy, hundreds of others exit with Bush

Washington. Any doubt that the Bush administration considered the directorship of the National Institutes of Health (NIH) to be a partisan post was removed last week when the White House informed its head, Bernadine Healy, and 650 other political appointees that they would be out of work after Bill Clinton's inauguration on 20 January as the country's forty-second president.

Letters declaring that their *pro-forma* resignations had been accepted were mailed on 13 January to every agency head serving "at the pleasure of the president", in other words, those appointed by George Bush for no fixed term and subject to confirmation by the US Senate. The sole survivor in science is the director of the National Science Foundation, Walter Massey, whose presidential appointment in 1991 extends for six years.

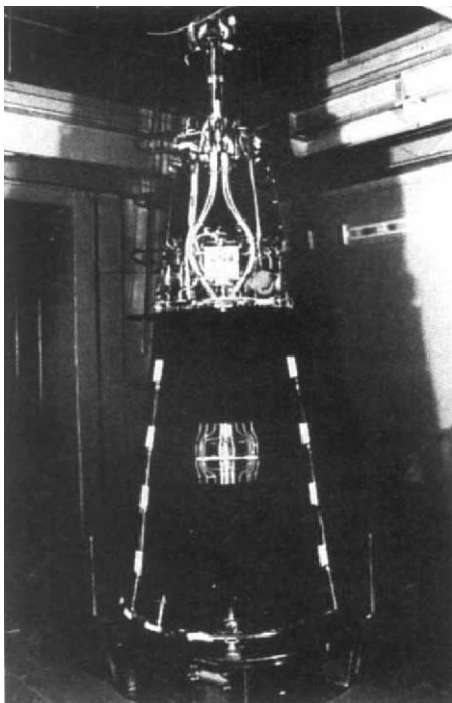
The action ends debate over whether Healy would be retained (see *Nature* **361**, 2; 1993), as well as resolving the status of David Kessler, director of the Food and Drug Administration, and Daniel Goldin, director of the National Aeronautics and Space Administration. Both had hoped to hang onto their posts, but it is thought very unlikely that Clinton will choose to renominate them or any other Republican appointees, given the large number of Democrats bidding for the jobs.

The decision to fire everyone was made after the Bush and Clinton camps were unable to agree on the number of political appointees to be held over, and puts pressure on Clinton to announce his selections as quickly as possible. Traditionally, political appointees submit their resignations to the incoming president, leaving him to decide who stays and for how long.

NIH officials profess surprise, saying that Clinton transition officials had told them that Healy would be exempted from any political housecleaning. At the same time, no professional society or special interest group campaigned to retain the NIH director, as has been the case during previous transitions. Their silence attests to the widespread unhappiness in the biomedical community over Healy's 21-month tenure, based on a belief that she ignored their interests while currying favour with her political bosses.

Speculation over her replacement is rampant but unrelated to the reality that the new president has more pressing business to attend to. But Bush's decision to end his political legacy with a stroke of the pen will doubtless increase the anxiety of researchers watching for the first tangible evidence of Clinton's attitude towards science.

Jeffrey Mervis



Topaz 2 reactor raises a ruckus.

RAC defers to NIH director on some gene therapy cases

Washington. The recombinant DNA advisory committee (RAC) of the National Institutes of Health (NIH) — a panel of lawyers, physicians and researchers charged with reviewing human gene therapy protocols — last week voted overwhelmingly to let the NIH director decide whether it is appropriate to bypass the committee in reviewing requests for the use of human gene therapy to treat individual patients.

The new measures were adopted at a hastily convened meeting of the RAC at which the NIH director, Bernadine Healy, defended her decision last month to deviate from normal procedures and grant permission to Ivor Royston of the San Diego Regional Cancer Center to treat a single patient with an otherwise untreatable brain tumour using an experimental gene-therapy procedure. Although RAC says that a full review remains the preferred course of action, an expedited review mechanism may be used when a patient's condition is too grave to wait for the full committee to consider it.

RAC members voted by nine to three, with one abstention, in favour of allowing the NIH director to make such decisions. Although most RAC members feel that NIH, like the US Food and Drug Administration, needs such a short cut, several members expressed concern that such a measure, by eliminating open discussion, will undermine the review process. They also feel that the field of gene therapy is still too new to forgo this level of public and scientific scrutiny.

A. Dusty Miller of the Fred Hutchinson Cancer Research Center, one of three RAC members to vote against it, complained that the RAC had "no room to move" because it acts in only an advisory capacity to the NIH director. "I don't like the idea of having a

political appointee review these proposals and make decisions on them", says Miller (see page 195). RAC member Abbey Meyers, executive director of the National Organization for Rare Disorders, fears that two sets of standards will now emerge: one for pressure groups with political clout, representing people with AIDS and cancer, and another for more "politically unimportant" diseases.

The issue arose in early October last year when Senator Tom Harkin (Democrat, Iowa) wrote to Healy on behalf of a 51-year-old former constituent suffering from a life-threatening stage-IV glioblastoma that was unresponsive to conventional treatments. Harkin asked Healy to consider granting Royston permission to treat the patient with an experimental gene-therapy procedure. Healy replied that there were not enough studies to consider using the therapy on a compassionate-plea basis and suggested that the patient be evaluated at NIH where an approved brain tumour therapy protocol was already under way. The patient rejected that approach because it would have required further brain surgery.

Royston's protocol was informally discussed at the RAC meeting on 4 December but not voted upon because adequate public notice had not been given, and the required review materials were incomplete. In view of the patient's deteriorating condition and in the absence of a mechanism for expedited review (RAC meets four times a year), Healy, after consulting NIH officials, gave Royston the go ahead on 28 December — the day that FDA also approved the protocol.

On 4 January, Royston began treating the patient with her own tumour cells, which have been genetically modified to express and secrete interleukin-2 (IL-2) in the hope of stimulating an anti-tumour immune response.

Diane Gershon

Tokyo University opens itself to outside scrutiny

Tokyo. In a move that could have profound long-term consequences for research at Japan's national universities, Tokyo University was host last week to a team of eminent Western and Japanese scientists carrying out an external review of the university's physics department. This is the first-ever such review in Japan and is part of a growing movement to make Japan's universities more accountable to outsiders.

The review was initiated by Tokyo University president Akito Arima and science dean Ikuo Kushiro (see *Nature* **360**, 403; 1992). Four reviewers live outside the country: Sydney Brenner of the University of Cambridge School of Medicine in Britain, David Pines of the University of Illinois, Yoichiro Nambu of the University of Chicago and Jean Audouze, science adviser to the French government. They were joined by several Japanese scientists, including Nobel prizewinner Leo Esaki, president of Tsukuba University; Minoru Oda, president of RIKEN; Junjiro Kanamori, president of Osaka University; and Hiroshi Takuma of the University of Electro-communications.

The reviewers, working in small groups, spent an afternoon and the next morning visiting laboratories doing research ranging from high-energy physics to genetic and molecular biological studies in biophysics. They also held a meeting attended only by younger researchers that, in the words of one department member, was "quite lively and lasted a long time". After reviewing financial matters, the team spent a day discussing what they had learned.

The reviewers were interested not just in science but also in grants, student recruitment and working conditions. Their presence has already had a "very good influence", says one department scientist, "forcing us to straighten up our thinking about what we are doing".

The reviewers are expected to submit their findings in about a month to Arima, who retires as president at the end of March. Arima says he does not know if the review process will be extended to other departments, but a senior official of the Ministry of Education, Science and Culture says that he expects Arima's successor to carry on the review process. University departments of lesser quality than the physics department at Tokyo University are expected to oppose the idea, but they may find themselves swept up in a tide of reform they cannot stop.

Researchers at Tokyo University do not expect immediate changes as a result of the review. "But in the long run I think it will affect the research system and the allocation of money", says one member of the department.

David Swinbanks

Press, Mullis win Japan Prize

Tokyo. Two US scientists have won this year's Japan Prize, Japan's answer to the Nobel prize. Frank Press, president of the US National Academy of Sciences, wins the ¥50-million (US\$400,000) award for his

contributions to seismological research and disaster mitigation. The other prize goes to Kary Mullis, a private consultant in nucleic acid chemistry, for his development of the polymerase chain reaction used for amplifying DNA sequences.

The Japan Prize was launched in 1985 to reward scientists and engineers who have made important practical contributions to society. The awards are made in different fields each year. Although intended to complement the Nobel Prize, which usually recognizes more basic research, the Japan Prize is not well-known outside Japan. The prize will be presented in Tokyo on 28 April.

David Swinbanks



Frank Press (left) and Kary Mullis

Mediation sought on biotechnology disputes

San Francisco. The biotechnology industry, confronted with indications that genetically engineered foods face a wary or even hostile public, has enlisted a mediation team to help it find common ground with its critics.

The first genetically engineered whole food, a tomato made by Calgene Inc., is likely to reach grocery store shelves later this year (see *Nature* 357, 352; 1992). But already chefs are vowing not to serve it, farmers are agreeing not to grow it and consumers are asking how it and other biotech foods will be labelled. Earlier this month, Campbell Soups announced that it had no plans to sell any product containing the tomato, which it helped to develop.

The Industrial Biotechnology Association has asked Resolve, a centre for environmental dispute resolution in Washington, to see whether its opponents would be willing

to talk face-to-face. "We were hoping that it might create a forum that would allow discourse at a slightly lower volume", says Richard Godown, president of the Washington-based trade association.

The association is concerned that the public might not accept the new products because of concern about the way the government regulates them. Last year, the US Food and Drug Administration (FDA) said that genetically engineered foods would not require any special labelling or safety testing unless there were indications that they might cause allergic reactions or pose safety concerns.

Environmental groups, convinced that the foods should be labelled, tested for safety and registered, urged their members to write to the FDA. Jeremy Rifkin, an outspoken industry critic, began organizing a boycott

and signed up thousands of chefs, growers and distributors.

The FDA has received 3,200 comments on its proposed regulations. About 85 per cent have asked that all genetically engineered foods be labelled, with 50 per cent also requesting premarket testing to prove safety. Food allergies were the concern of 40 per cent, while 10 per cent raised ethical or religious issues. The FDA is considering an open meeting to talk about the labelling question and is meeting experts on food allergies, said James Maryanski, biotechnology coordinator for the agency's Center for Food Safety and Applied Nutrition.

FDA officials also have agreed to an initial meeting with Resolve, which is contacting environmental groups such as the Environmental Defense Fund and the National Wildlife Federation. Rifkin has not been approached. Calgene is also a participant.

Resolve, a subsidiary of the World Wildlife Fund, helped to bring about the National Wetlands Policy Forum that led to a government policy stipulating no net loss of wetlands. For five years, it has conducted a quarterly roundtable meeting on the ecological effects of pesticides with representatives from government, industry, universities and the environmental movement.

The organization typically convenes a group of about 20 people holding diverse views on a particular issue and tries to reach a consensus. The result can be policy recommendations, agreements on joint research or even a draft of new federal rules. In the case of genetically engineered foods, new regulations would not be possible because the FDA did not initiate the process.

Resolve expects to complete a feasibility study within a couple of months to decide whether the project should proceed, what its goals would be and who would fund it. Potential participants are optimistic.

Sally Lehrman

US keeps promise on biodiversity

Washington. Outgoing President George Bush this week was expected to sign an executive order creating a National Biodiversity Center to serve as a repository for scientific data on biodiversity within the United States and its territories. The order, coming in the final days of an administration with a reputation for dragging its feet on environmental issues, directs the Smithsonian Institution to take the lead in working out an agreement among federal agencies to establish and fund such a centre.

At present the US government has no clearinghouse for biodiversity information. Taxonomic and other data on species, habitats and ecosystems exist in many places, including museums, universities, government agencies and conservation groups. In its simplest form the new centre would set up an electronic network linking the different databases; a more elaborate plan would be a single large database at a location yet to be determined.

The executive order designates a committee of at least a dozen federal agencies to determine the scope and structure of the new centre, which could be in operation within a year.

The centre is not expected to require the construction of a new facility. It would neither collect data nor do biological surveys. "There's a strong argument for keeping the science separate from the policy", says Thomas Lovejoy, assistant secretary for external affairs at the Smithsonian and a leading figure in negotiations to create the centre.

In fact, one reason the Smithsonian was selected is its reputation as a nonpartisan research agency. "Biodiversity is still a politically touchy issue, and they [the White House] wanted to put the centre somewhere

with the least possible political content", says Peter Jutro, who heads the biodiversity research programme at the Environmental Protection Agency.

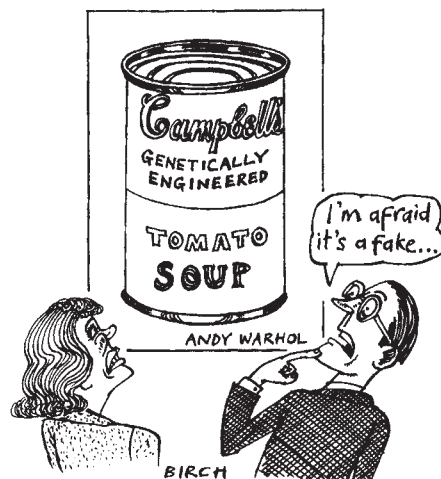
Both the centre and an international workshop on biological surveys held last week in Washington fulfil promises made by the Bush administration during the environmental summit held last summer in Rio de Janeiro. Although the United States did not sign the convention on biological diversity at the summit, it endorsed the idea of establishing centres to coordinate scientific data-gathering within individual countries and offered to host the first international workshop to set standards for coordinating biological surveys.

White House officials wanted to hold the workshop, involving scientists and administrators from several US government agencies as well as representatives from 12 other countries, before the end of Bush's term of office on Wednesday this week. Its recommendations will go to the United Nations Environment Programme and to other organizations working on biodiversity issues.

Participants at the workshop were concerned that museums and private institutions might be reluctant to share their data, particularly with commercial interests that might use them for profit. "Some of the best experience in this area is in private zoos, herbariums and organizations like the Nature Conservancy", says Jutro. "We have to have a big tent."

Although Bush was expected to sign the order creating the centre before leaving office on 20 January, Clinton also supports the idea, says Jutro. "I think it will be followed up enthusiastically by the next administration."

Tony Reichhardt



Forecasters seek better data on work patterns of scientists

London. An announcement last week that employment prospects for graduates in Britain are worse than they have been for a decade has highlighted the importance, not confined to Britain, of matching the supply of scientists to the demands of research and industry. But attempts by governments in the 1960s and 1970s to use central planning to achieve this goal went badly wrong, often with disastrous results. So what can be done to minimize the chances of a serious mismatch between a country's scientific needs and its labour force resources?

The answer, according to forecasters attending a meeting in London last week organized by the Science Policy Support Group (SPSG), is to shift the focus of the exercise. Recognizing that most forecasting relies on little more than the dubious practice of extrapolating existing trends, participants agreed that emphasis should be placed not on prediction but on a better understanding of the present.

Attempts to do this, however, are plagued with problems. One is the paucity of relevant data (combined with the fact that the available data are seldom sufficiently detailed to be useful to policy makers). A second is that data collected in one country are not necessarily comparable to those collected in another, making international comparisons difficult.

The Commission of the European Communities (EC) confronts both problems in designing programmes intended to boost research and technology. "Statistics and indicators on human resources in science and technology are very incomplete, and of variable quality and comparability", says Ian Perry, who manages statistics in the EC's

director general for science, research and development. "The quality, coverage and comparability of data need to be radically improved."

Britain has already abandoned the idea of central planning to produce a perfect match between supply and demand, but it is clear that the market alone cannot deliver the best solution. What is needed is better information about such characteristics as the precise number of scientists and engineering in the workforce and how they are used.

The Office of Science and Technology is therefore planning to develop a monitoring system that would provide a central, rolling database of scientists in employment. One of the models being studied is that used by the US National Science Foundation in its biennial science indicators series.

Aware of the advantages of being able to make international comparisons — and at the same time of some of the limitations of aggregated statistics — the Organization for Economic Cooperation and Development (OECD) in Brussels has encouraged its member countries to carry out more detailed analyses of their scientific workforces.

However, even OECD statistics have their limitations. For example, the organization does not yet provide statistics on the number of women engaged in science because officials believe that the data are not good enough. Another area is the international flow of scientists. Although most countries keep aggregate data on foreign scientists working within their borders, few know the number leaving to work elsewhere.

David Dickson

South Africa to form nonracial science academy

Cape Town. Three South African scientific societies organized along racial lines have agreed to form one academy embracing the natural sciences, humanities and engineering. The new nonracial body, to be called the Academy of Science of South Africa, has the support of the three existing academies: the predominantly English-speaking Royal Society of South Africa, the Afrikaans-speaking Suid-Afrikaanse Akademie vir Wetenskap en Kuns, and the (black) Science and Engineering Academy of South Africa.

The new academy is neither an amalgamation of nor a replacement for the existing science academies, according to Reinhard Arndt, chairman of the academy's temporary nominations committee and president of the Foundation for Research Development, a government granting agency. Members will be chosen from those who have made outstanding research contributions or those "demonstratively capable scientists who are in a position to promote the academy's objectives", says Wieland Gevers, a former president of the Royal Society of South Africa. The election process is nothing if not complicated.

Each of the three existing academies will provide a dozen people, not necessarily members of the academy concerned, to serve on a facilitating committee to elect the first 60 members of the new body. They are not themselves eligible for election. These 60 members will then elect 40 more, with members of the facilitating committee now eligible. These 100 founding members will review the constitution drawn up by the facilitating committee and elect officers and additional members as they see fit.

Members will be asked to vote for or against each candidate, or to register a neutral vote. The candidates with the largest number of affirmative votes will be elected, and anyone with more than 25 per cent negative votes will be excluded, allowing an existing academy to defeat someone if its members vote as a bloc.

Prospects for funding the new academy are uncertain. The government has awarded it a very modest initial grant of R100,000 (US\$32,000) but has made it clear that the new body must look to the private sector or its own activities, such as publishing textbooks and other scholarly materials, for funding. Of the existing academies, only the Afrikaans one receives a state grant. Another unresolved matter is the location of the academy, a potentially contentious issue in a country with strong regional tendencies.

Michael Cherry

Japan reacts to special issue

Tokyo. Recommendations for reform of Japanese science, contained in a recent special issue of *Nature* on Japan (see 359, 573–582; 1992), have caused quite a stir in Tokyo extending to the highest levels of government.

Last month, Minoru Kawashima, a member of the Social Democratic Party of Japan, asked for the government's reaction during a meeting in the Diet of the lower house committee for science and technology, comprised of representatives of both the ruling and opposition parties. The questioning follows considerable coverage in the Japanese media.

The *Nikkei Sangyo Shimbun*, a leading daily newspaper for industrialists, ran a series of articles and translated the main article

from *Nature* ("Reforming Japan's science for the next century") into Japanese. Last month, *Kagaku Asahi*, a popular science monthly, published a roundtable discussion by three government officials and a university professor on both *Nature*'s special issue of 15 October and one that appeared the following week in *Science*.

Tomonori Kudoh, director of the university education division of the Ministry of Education, Science and Culture, replied to Kawashima's question by saying that "we think from one point of view *Nature* was right on the mark on several matters, and so do many university professors, but [the article] was rather one-sided. However, we receive those recommendations humbly."

David Swinbanks

Structural chaos in Italy

SIR — Your cover for 12 May 1983 symbolically showed Etna erupting splendidly, and your caption contained the phrase “Italy, perhaps the only country in Europe where chaos is structural”; in that issue you published a review of science in Italy under the title “Can order spring from chaos?”¹ We wish to reply that, as far as we are concerned, the answer is still unfortunately “No”.

In 1982, *Nature* commented: “Italian science policy appears at last to be getting into gear . . . politicians here have reached a consensus . . . that research is necessary to pull Italy out of its economic crisis”². You later reported³: “Scientific hearts are beating faster in Italy . . . as real hopes emerge of genuine reform of Italy’s major but chaotic research council, the Consiglio Nazionale delle Ricerche (CNR)”.

The CNR has in fact spent relatively very large sums of money on us (an institute for plant virus research), providing new laboratories and an excellent new greenhouse facility, and making big investments in equipment and materials. This is in line with a recent report by the National Committee for Agricultural Sciences that our level of applied and fundamental research is *ottimo* (excellent, the top rating) and that we work with *grande impegno e proficuità* (great commitment and productivity).

Nevertheless, over a period of seven years, there have been no new scientific staff appointments, despite the loss, through retirement or transfer, of four researchers and three technicians from a total of 17 research personnel. The average age of our greying scientists is 51. We are also losing bright young research workers who have trained here and abroad for up to five years, because there is no regular career structure open to them. Thus a really impressive investment by the CNR in buildings and materials has been matched by a relentless rundown in our capacity to profit from this investment.

On 20 July 1990, 10 January 1991 and 21 January, 20 March, 7 September and 15 September 1992, our director wrote to CNR headquarters in Rome underlining various aspects of the situation and repeating our requests to have at least some replacement or new blood, to match the investment in hardware, and the favourable verdict of the peer review committee. There has been no reply to any of these letters, except that the letter of 20 July 1990 was answered on 25 September 1991 (14 months later) by a request for clarification but with no response to our queries. It thus appears impossible to conduct any coherent dialogue with the CNR.

You recently published a letter from Gertrud Lund, recipient of a fellowship in another CNR laboratory, saying that the CNR had sequestered her salary and bench fee, which represent European Communities funds⁴. We can believe her story, as the CNR, for more than a year, has pocketed about 100 million lire (£44,000 or ECU64,000) that we earned through outside contracts. This money would have enabled us to support a graduate student and a technician. We suppose that the sum will eventually be released to us (without interest) but by then the party will be over, and the work, already paid for, will not have been done. We still have no idea when this money or indeed a second tranche due in 1993 will be released.

R. G. Milne

on behalf of 32 personnel of the
Istituto di Fitovirolologia

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Procedure for gene patents

SIR — Your leading article (*Nature* **359** 348; 1992) enthusiastically applauding the rejection by the United States Patent and Trademark Office (PTO) of the Venter *et al.* gene patent application (*Nature* **359**, 263; 1992) was, perhaps, premature, and reflects an incomplete understanding of patent prosecution before the PTO.

All that has happened so far is that a lower-level patent examiner has made a routine first-round rejection of the application’s claims on grounds of a lack of utility and novelty, and obviousness. First-round rejections in the PTO are made in virtually all cases, particularly in the biotechnology examining group handling this application, and particularly on grounds of obviousness (to some examiners, Noah’s Ark makes obvious all subsequent patent applications claiming a boat). It is not necessarily significant. The rejections are based either on the examiner’s lack of acumen or his or her wish to make the applicant actively defend the claims.

Contrary to what you say, an appeal, which is a formal proceeding before the Board of Patent Appeals and Interferences (the PTO’s highest appellate tribunal), is *not* the next step. If, for policy reasons, the National Institutes of

Health (NIH) decide to go ahead with the prosecution of the application, NIH’s patent attorneys (usually a private sector patent law firm under contract to NIH) will request reconsideration by the lower-level examiner and will argue against (that is, will “traverse”) the rejections.

Only if the examiner again rejects all (or some) claims may NIH appeal to the board to review the rejections. Even if NIH fails to persuade the board to reverse the decision on substantive grounds, or if the board makes a political rather than a legal decision, NIH has recourse to civil litigation before federal courts at three levels.

So, there is a long way to go, and your cock may be crowing prematurely.

Melvin Blecher

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Conflict of interest

SIR — Having read your article about the code of practice in a case of a referee’s conflict of interest (*Nature* **360**, 205; 1992), I must say that I agree with John Maddox that “lapses from strict propriety are exceedingly rare”, but close examination of the problem would reveal a much bigger problem than suggested in the article. Unfortunately, there were no suggestions as to how the peer system could be improved.

I would like to suggest the installation of a feedback loop in the existing peer review system that connects the referee with the author. In such a system, the authors would have the right to have the name of the referee disclosed after an appropriate time period of perhaps 3–5 years. This would make the referee aware that the author will know his identity and push him to give a fair judgement or to declare a conflict of interest and it could restrain the referee if he/she is not just an altruistic and selfless judge.

A period of 3–5 years is long enough in modern science for conflicts of interest to be manifested in the published work of both sides, yet short enough for the case, if there is one, to be discussed with author, referee and editor. On the other hand, most rejected authors will not be interested in what happened to their manuscripts after such a long time, having published their data elsewhere.

Publications in science are too important to let the referees go ‘unrefereed’.

Albrecht Müller

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Street pigeons in Basel

SIR — Because of overpopulation, street pigeons (*Columba livia forma urbana*) have become a plague in most cities of the world. They cause a variety of health and environmental problems and themselves suffer from poor, 'slum-like' living conditions¹. Attempts to reduce the populations, whether by killing or by treatment with sterilizing agents, have not been successful. A prohibition on pigeon-feeding, issued in 1978 in Basel, failed because of public protest and simple ignorance. The government therefore asked the department in which I work to analyse the causes scientifically and find a practical solution.

The street pigeons in Basel depend upon human 'pigeon-feeders' who provide the large food base that is principally responsible for the overpopulation². Preliminary studies demonstrated that a population reduction is obtainable only by a reduction of the ecological capacity of the system². This means the large feeding base is the system's limiting factor. Killing pigeons only rejuvenates the flocks and has no long-term effect upon the population size^{3,4}.

In 1988 and 1990 we launched our information campaigns "Pigeon Action I and II" (see figure) in collaboration with the Society for the Protection of Animals of Basel. We tried to explain the complicated ecological relationships between the large food base and the resulting overcrowding. We asked the public by pamphlets, posters, radio and television to stop or at least to limit their feeding with the slogans: "Feeding pigeons is animal cruelty", and "Protection of animals is: not feeding pigeons!"

It seemed important not only to prohibit feeding but to offer an alternative

for pigeon friends. We therefore built nine controlled and well-kept pigeon lofts to house a small but healthy population. Nearby, we developed designated man-pigeon encounter areas where the feeding of the pigeons is allowed. We yearly remove about 1,200 eggs and so can keep these flocks small.

We weekly observed the size of 13 control flocks in different parts of Basel. Within 50 months, the total population of these flocks was reduced by 50 per cent (see figure). This corresponds to a reduction in the total Basel pigeon population to 10,000. Our aim is a small and healthy stock of about 5,000 street pigeons, a goal we hope to reach in the next few years.

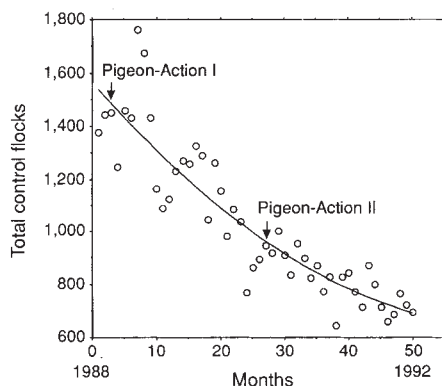
Our Pigeon Action has been well-received nationally and internationally. We have found a satisfactory change in the mental attitude of the public. Feeding pigeons has become taboo and only a few incorrigible people continue.

If man's activity is the cause of an ecopathological effect, then the solution must be sought in a change of public attitude rather than in a change in the affected ecosystem. Our results demonstrate that by changing mental attitudes, the population of the street pigeons can be permanently reduced, to the benefit of both the animal and its environment.

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Monthly total street pigeon population (in 13 control flocks). The start dates of the two pigeon campaigns are indicated as Pigeon-Action I and II. The regression line shows the decrease in number of the pigeons within 50 months from 1,400 (summer average 1988) at the beginning to 708 (summer average in 1990) by the end of the investigation.

Body heat

SIR — While the DREADCO biochemists are searching for an alcohol substitute to warm the lost and snow-bound¹, I would suggest they investigate the body's own heat generating mechanism, which has been known since antiquity and which was reported in *Nature* ten years ago by Benson and others².

The production of 'magical heat' by breath control techniques has been known since prehistoric times³ and it is practised in the form of *g Tum-mo* yoga in Tibet³ and India² and in other religious practices of the indigenous peoples of Africa, Asia, Polynesia, Europe and North America⁴. It has been suggested that these practices increase the skin temperature due to an increase in car-

diac output concomitant with a decrease in hepatic and renal blood flow, resulting in an increase in flow in the cerebral and peripheral vessels⁵.

Finding the putative neuropeptide (perhaps a thyroxine analogue) that specifically increases the parasympathetic tone in these individuals, or developing a specific blocking agent to the adrenergic receptors of the sympathetic system, may be the safest and most practical way of bringing DREADCO's 'Body Heat' to the market place.

August L. Reader

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Thatcher right?

SIR — You are, of course, entitled to your liberal bias and the Opinion section is the proper place for your editorial comments, but consider, if you will, how your "taboo" remarks might sound to a conservative reader.

In the first paragraph, you seem to take delight in spanking Margaret Thatcher for not using money wrung from British taxpayers to fund a survey of sexual habits that you think is so smashing. In the second column you explain that one of the reasons for her disapproval was scepticism about the veracity of the prospective survey data. Then you admit that "[t]he premise is correct" in the very next sentence!

Now, just what was it that Mrs Thatcher was mistaken about? One of her objections was, by your own admission, valid. Even if it hadn't been, she undoubtedly had other reasons for her frugality. For example, she may have wondered, as I do, just how much more data we need to identify the behaviours that risk transmission of AIDS. Or, how much more money we must extort from those not at risk to attenuate the affliction of those who, at their own discretion, are?

This conservative American reader, for one, would be delighted to have a government as concerned as Mrs Thatcher about how much tax money is being spent chasing a treatment for a disease whose overwhelming cause is volitional behaviour that is at best irresponsible and at worst degenerate.

William P. Best

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Galactic origin of cosmic rays

Measurements of γ -rays from the Small Magellanic Cloud seem to have ruled out the possibility that cosmic rays are uniformly spread through extragalactic space, but do not say much more about their origin.

So that's it then. Cosmic rays are not a universal phenomenon, spread through intergalactic space as well as other galaxies, but those that it is possible to observe near the surface of the Earth are predominantly the products of this Galaxy alone and, apart from a little leakage at the edges, are confined to it.

Declarative statements such as that, on such great issues, cannot often be made these days. As will become apparent, even this can bear a little qualification. Direct proof of any such assertion naturally lies a long way off; there are as yet no plans for launching intergalactic measuring devices, for example. But may it not be possible to tell something about the general distribution of cosmic rays by looking for evidence of their presence (or absence) in other galaxies?

The obvious difficulty is that the other galaxies are so far away. The more serious difficulty is to know how it would be possible to tell whether there are cosmic rays in other galaxies. Searching for cosmogenic carbon or some isotopic consequence of a flux of cosmic rays comparable with that at the Earth will remain a hopeless task, at least until somebody finds an extragalactic (or, for that matter, a galactic) Earth-like planet. Some other chain of inference is called for.

The first clear recipe for deciding between a galactic and metagalactic origin for cosmic rays was provided more than 20 years ago by V. L. Ginzburg (who is still attached to the Lebedev Institute in Moscow). In 1972, he suggested (while disclosing a prejudice in favour of the galactic hypothesis) that a measurement of γ -rays from the Large and Small Magellanic Clouds would provide a crucial distinction between the two possibilities (*Nature Physical Science* **239**, 8; 1972).

How does the argument go? The energetic particles of cosmic rays inevitably collide with interstellar gas, producing energetic γ -rays. But the quantities of interstellar matter in the Magellanic Clouds had already been inferred from measurements by radioastronomers of neutral hydrogen in them. Ginzburg then simply calculated the flux of γ -rays expected from the two satellite galaxies on the two hypotheses.

On the metagalactic view, the γ -ray flux should be that produced by a flux of cosmic rays similar to that near the Earth; otherwise, it would be smaller. Restricting the argument to γ -rays more energetic than 100 MeV (to avoid low-energy stellar processes), Ginzburg concluded that, if his preferred

galactic hypothesis were correct, the combined γ -ray flux from the Magellanic Clouds would be only a third of that expected on the metagalactic view — and that the output of the two clouds should also be significantly different.

Ginzburg's expectations have now been amply confirmed by data gathered by the Compton Gamma Ray Observatory, a satellite launched in 1991 from the United States and managed from the Goddard Space Flight Center. The essence of the instrument is a γ -ray telescope built so as to detect γ -rays much as that job was done in the old cloud-chamber days, by using parallel sheets of a material such as lead to convert them into electron-positron pairs. The telescope provides information about the direction of the γ -rays received as well as their energy. Calibration has shown that the background noise is at least an order of magnitude less than the signal expected from intergalactic space.

The inference that cosmic rays have a galactic origin (and that the Magellanic clouds produce their own), or at least that the metagalactic hypothesis does not stand up, is based on six weeks worth of data gathered when the telescope was pointing towards the Small Magellanic Cloud (Streekumar, P., *et al.*, *Phys. Rev. Lett.* **70**, 127; 11 January 1993). The authors, of whom there are 16 (four from the Max Planck Institute of Extraterrestrial Physics at Garching, Munich), have carefully subtracted from the measurements the γ -ray flux expected from the Galaxy and from the local Compton scattering of low-energy photons by energetic cosmic γ -ray particles.

The outcome is that the measured flux of γ -rays more energetic than 100 MeV is reckoned not to be greater than $0.5 \cdot 10^{-7}$ photons per square centimetre per second; this estimate allows for the uncertainties of the measurement at a confidence limit of 95 per cent. In other words, the chance that the output from the Small Magellanic Cloud exceeds this limit is 5 per cent. By contrast, on the metagalactic view, the expected flux from the cloud should have been $2.4 \pm 0.5 \cdot 10^{-7}$ photons $\text{cm}^{-2} \text{s}^{-1}$. The two numbers differ by a factor of five and, although the error of the estimated output on the metagalactic view may seem uncomfortably large to some, few will doubt that it is large enough to carry the day for the view that cosmic rays have a galactic origin.

So far as it goes, this outcome is comfortable enough. Ginzburg, at least, should be pleased, not least for his clear statement 20

years ago that instruments then being designed for satellite use would have sufficient sensitivity to discriminate between the two possibilities. But, inevitably, things have become a little more complicated since 1972, not least because of the doubts astronomers have thrown on the intrinsic stability of the Small Magellanic Cloud.

That something fishy is going on seems well established. Radioastronomy suggests that the satellite galaxy has two components with different velocities, optical observations have suggested that it is thinner along the line of sight than it should be if it is to be stable, while there is a tidal theory involving a close encounter with the Large Cloud less than 100 million years ago that provides a means by which it may have been rendered unstable. These arguments are relevant, because the possibility that the Small Magellanic Cloud may be in the process of disintegrating is the origin of the large uncertainty in the γ -ray flux expected on the metagalactic view.

For what it is worth, measurements with the same instruments of the Large Magellanic Cloud, believed to be stable enough, yield only an inconclusive result. The measured flux is less than that expected from the metagalactic view, but given the uncertainties of measurement and calculation, the difference is insignificant. There the inferred intensity of cosmic rays, which exert an expansionary (or counter-gravitational) pressure within a stable galaxy, seems comparable with that near us.

Meanwhile, it will have been noted that this development may have ruled out one hypothesis whose implications for the energy balance of the Universe have always made some people uncomfortable, but has not pointed to the true sources of cosmic rays. At least the metagalactic camp has been able to claim that quasars, radio galaxies and other distant but poorly understood objects were plausible sources of energetic particles. If the major sources of energetic particles must now be sought within the Galaxy, there will be some difficulty in adding up the effects of supernovae, collapsing binaries, γ -ray bursters and the like to yield the observed flux of cosmic rays. And while Fermi's mechanism of the acceleration of low-energy particles to cosmic γ -ray energy by random magnetic fields remains a possibility, it has the drawback of being almost untestable.

But people will at least now know where to look.

John Maddox

Becoming B cells

Stephen Desiderio

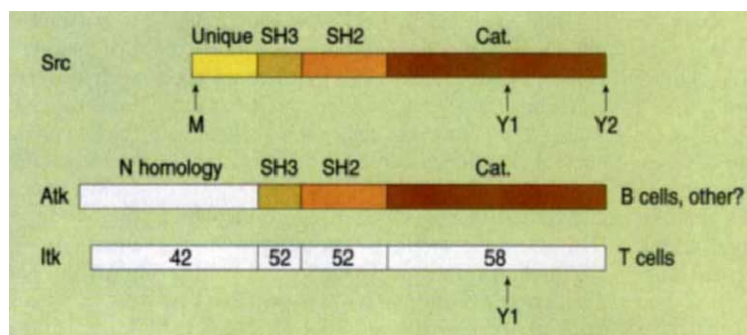
EVERY so often, adventurers approach a problem by an unworn path, only to emerge at what seems to be, at least at first, a familiar place. Such convergences may be merely reassuring, but the happiest ones can be stimulating. An encounter of the latter kind is found in the paper on page 226 of this issue¹, in which Vetrie *et al.* show that the gene involved in X-linked agammaglobulinaemia (XLA) encodes a hitherto unknown protein-tyrosine kinase.

Bruton's report² of a child with recurrent bacterial infections and lack of serum immunoglobulin (Ig) was the first description of what later came to be recognized as XLA³. This uncommon disorder affects boys, who are generally identified by their unusual susceptibility to bacterial infection. Susceptibility is not limited to bacteria, however, as persistent viral and parasitic infections are also observed. Boys with XLA are unable to mount antibody responses, and all classes of serum Ig are absent.

The disease results from a block in the development of B lymphocytes. The immediate progenitors of B cells, pre-B cells, express Ig heavy chains but not light chains. Upon assembly and expression of functional light-chain genes, pre-B cells give rise to B cells, which express surface-bound Ig. After antigenic stimulation, B cells can differentiate into antibody-secreting factories — plasma cells — that secrete several thousand Ig molecules per minute. This terminal stage in differentiation generally requires the assistance of T cells. In XLA patients, plasma cells are essentially absent and circulating B cells are rare. The number of pre-B cells in the bone marrow, however, is normal, indicating an impairment in the transition from pre-B to B cell⁴. T cells in XLA patients are apparently unaffected, and tests of cell-mediated immunity are normal. The selectivity of the XLA defect for B cells is underscored by the efficacy of replacement therapy: repeated injection of immunoglobulin — as Bruton observed 40 years ago — can greatly reduce the frequency of life-threatening illness.

Given that B-cell development depends at many points on signals from other cell types, including stromal cells and T cells, a B-cell deficiency could result from defects intrinsic or extrinsic

to the B lineage. XLA is associated with an intrinsic defect^{5,6}. In females, one of the two X chromosomes in each somatic cell becomes randomly inactivated during embryogenesis. The B cells of proven XLA carriers, unlike other types of cells from the same individuals, show a lopsided pattern of X inactivation; the inactive X is generally the one that carries the XLA mutation. When the inactive X is the one that carries the normal XLA locus, B-cell development is apparently blocked.



Comparison of Atk, Itk and Src. Unique, SH3, SH2, catalytic (Cat.) and amino-terminal homology (N homology) regions are indicated. Numbers represent the percentage of amino-acid similarity between corresponding regions of Itk and Atk. M, Y1 and Y2 indicate myristoylation, autophosphorylation and negative regulatory sites, respectively.

Thus the XLA product is made in B-cell progenitors and is required after the emergence of pre-B cells. A functional product is not absolutely required for assembly of antibody genes or synthesis of heavy and light chains, because surface Ig is expressed on some B-cell lines derived from XLA patients. Without obvious candidate genes or biochemical insight into what is going on, it has seemed clear that the most direct way to identify the XLA product would be by 'positional cloning' — that is, by its map position.

The herculean strategy is by now familiar, at least in outline⁷. Begin by scouring affected pedigrees for polymorphisms that are linked to the disease locus; assemble a set of genomic DNA clones spanning the interval to which the locus maps; identify transcripts derived from this region; and screen the transcripts for mutations that correlate with disease. A pair of polymorphic DNA markers had been linked to the XLA locus, localizing it to an interval on the long arm of X. Additional linked DNA markers were found; one of these, DXS178, showed no recombination with XLA, indicating that it was very near. A yeast artificial chromosome clone spanning DXS178 was therefore used to fish out candidate B-cell complementary

DNA clones. Two of the clones detected DNA rearrangements in XLA families and were derived from the same transcript. In a large proportion of affected families, the locus encoding this transcript was altered in all family members known to have the mutant XLA gene. Two of these mutations were intragenic deletions, providing compelling evidence that the locus is involved in XLA.

The associated transcript appears to encode a protein-tyrosine kinase, dubbed Atk (for agammaglobulinaemia tyrosine kinase). The proposed enzymatic activity of Atk is apparently required for normal B-cell development because, in two XLA families, single amino-acid substitutions were detected at sites that are critical for kinase function. Atk lacks an obvious transmembrane segment and appears to be wholly intracellular.

While the authors interpret Atk to be a member of the Src kinase family, to me things look a bit different (see figure). Atk does share some structural features with Src kinases, including a putative autophosphorylation site and two regions, SH2 and SH3, that mediate specific protein-protein interactions. But whereas the activity of Src kinases is negatively regulated by phosphorylation of a conserved tyrosine residue near the carboxy-terminus, Atk lacks this regulatory tyrosine. In addition, all Src kinases have a consensus sequence for myristoylation near the amino-terminus, but the longest predicted version of Atk lacks such a sequence, although a potential internal start site could perhaps be myristoylated. These differences suggest that Atk may turn out to differ somewhat from the Src kinases in its regulation and intracellular localization.

In fact, two other mammalian proteins — Tec⁸ and the newly described Itk⁹ — are remarkably similar to Atk; together they appear to define a new family. All three kinases show restricted patterns of expression. Tec is expressed predominantly in liver; Itk specifically in cells of the T-lymphocyte lineage. The preliminary data for Atk suggest that it is expressed in B cells and perhaps some other cell types, but not in T cells. Like Atk, Tec and Itk are distinguished from the Src family by their lack of an amino-terminal myristoylation consensus sequence and carboxy-terminal regulatory tyrosine. Another distinction is seen in the catalytic region; where Src kinases carry the sequence HRDLRAAN (residues 384 to 391 of p60^{c-src}), all other

known protein-tyrosine kinases, including Atk, Tec and Itk, have HRDL-AARN. A striking feature of these kinases — especially evident for Atk and Itk — is the presence of additional homology at their amino-termini (see figure). Might this region bind common upstream or downstream effectors unrecognized by the Src kinases? The similarity between Atk and Itk provokes the thought that Itk may play an analogous role in T-cell development.

It now becomes possible to study the XLA defect at the biochemical level, starting with Atk and working upstream or downstream. A bewildering abundance of protein-tyrosine kinases have been implicated in B-lymphoid signal transduction, but Atk arrives with an advantage: a phenotype. Atk is not the first protein-tyrosine kinase to have been implicated in B-cell development, but it is the first in which a null mutation has been shown to result in a profound, selective developmental block. This guarantees that studying the function of Atk will illuminate a crucial developmental step.

Ironically, an early task may be to create an Atk-deficient mouse. The availability of a mouse model for XLA, in addition to its value in evaluating therapies, would permit a finer analysis of the developmental defect than can be achieved with human subjects. Eventually, by interbreeding Atk-deficient mice with mice deficient in other putative B-cell signalling proteins, functional molecular interactions might be revealed or verified.

Finally, the work has diagnostic and therapeutic implications. It will now be possible to detect carriers and to perform prenatal tests by direct analysis of mutations. Because the B-cell lineage is selectively affected, it is conceivable that XLA may one day be treated by gene-replacement therapy. The fact that protein-tyrosine kinases have also been implicated in tumorigenesis, however, will temper enthusiasm for this approach until more is known about normal function and regulation of Atk. □

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The ups and downs of X-rays

Jules P. Halpern

PERHAPS the greatest impediment to understanding active galactic nuclei, the black-hole driven cores of quasars, Seyfert galaxies and blazars, is the conspicuous absence of periodic signals in their emissions. In contrast, numerous exotic, compact objects in our Galaxy, such as pulsars, accreting white dwarfs and neutron-star binaries, have periodic behaviour, enabling astronomers to determine their size, mass and history. But now, on page 233 of this issue¹, Papadakis and Lawrence report the discovery of quasiperiodic (but not strictly periodic) variations in the X-ray emission from a well-studied Seyfert galaxy, NGC5548. Using archival data from the EXOSAT satellite, they find a signal with frequencies near 0.002 Hz, corresponding to quasiperiods of around 500 seconds.

In contrast to the strictly periodic signals that commonly arise from stellar rotation, pulsation and orbital motion, quasiperiodic oscillations (QPOs) are less precise. Characterized by slight wandering in frequency or phase, and often wider excursions in frequency which are related to the variable intensity of the source, QPOs are seen from accreting white dwarfs and neutron stars in binary systems. In most cases, the signals are thought to arise from unstable processes in the inner part of a disk of material, a differentially rotating fluid, orbiting around and dragged down onto the central compact object. The oscillations certainly signal important details about the accretion phenomenon, so that considerable effort has gone into interpreting them.

It was once thought that accreting black holes would not give QPOs because, unlike neutron stars, they lack a stellar surface onto which to anchor a rotating magnetosphere; this is thought to be the crucial element in the generation of QPOs from neutron-star binaries. But over the past year, QPOs have been seen from a number of black-hole candidates in our Galaxy, including the celebrated Cygnus X-1^{2,3}, and from a number of hard X-ray transient sources⁴⁻⁶ which are looking more and more likely to be black holes. Even so, the frequencies are only about 0.04 Hz, rather low to be coming from the inner accretion disk, so that their origin is still a puzzle.

When reporting their studies of accreting, compact objects, X-ray astronomers often credit themselves with the ability to observe phenomena that arise closest to the origin of power. But the detection of 0.002-Hz QPOs from NGC5548 is an occasion for anxiety as well as for congratulations, because it evokes the

spectre of a source that is implausibly small. The period of these QPOs is so short that the conventional interpretation in terms of orbital motion in the accretion disk around a black hole cannot accommodate a mass greater than 10^6 solar masses. This value would be rather small for an active galactic nucleus and conflicts with the 10^7 – 10^8 solar masses that have been attributed to the cores of NGC5548 and similarly luminous Seyfert galaxies from detailed spectroscopic studies.

Acoustic oscillations in the inner accretion disk could be invoked, but would not help, as their frequencies are even lower than the orbital frequencies⁷. Magnetic reconnection in the accretion disk is a similarly unappealing mechanism, as it is tied directly to the orbital period⁸. If the central body were a rapidly spinning black hole — a so-called Kerr black hole — the frequency would be less of a problem: for a given mass, the spin allows the inner edge of the accretion disk to reach a smaller radius, and so to have a shorter orbital period. In this case, 10^7 solar masses would be allowed.

It is instructive to contrast the case of NGC5548 with that of NGC6814, a Seyfert galaxy that is unique owing to strict periodicity in its X-ray emission. NGC6814 has a period of 12,100 seconds which is coherent over one year⁹, and is compatible with orbital motion of an object in the accretion disk at a distance of 50 gravitational radii from a 10^6 -solar-mass black hole. NGC6814 is easier to accept as an object of a million solar-masses because there were several previous indications that it might be unusually small. Long before the detection of its periodic oscillations, NGC6814 was known as a rapidly variable Seyfert galaxy, and one of the least luminous. X-ray emission-line variability on a timescale of less than 300 seconds¹⁰, and an X-ray luminosity less than a tenth that of NGC5548, marked NGC6814 as a prime target of any search for short-period oscillations. But NGC5548 enjoys no such distinction; its X-ray properties, apart from the QPOs, are rather ordinary. If the case of NGC6814 were to predict anything about variability in the X-ray emission from other Seyfert galaxies like NGC5548, it is that periods of days or longer should be expected. Given the difficulty of discovering such long periods in the sparse data that are available, it should surprise no one that periodic signals are not found more commonly.

Therein lies the dilemma in Papadakis

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and Lawrence's results. Difficult as it is to explain the strict 12,100-second period of NGC6814, any mechanism carefully contrived for that purpose would be of little use in interpreting the more rapid quasiperiod of NGC5548. In the absence of a plausible theory, sceptics often focus on the reliability of the observations. How certain should we be of the QPO feature? The authors have done a thorough job in investigating this question, but it is still a dangerous business, assessing the significance of a feature that is permitted to vary in frequency and breadth as well as in intensity. On the one hand, its transient nature makes verifying the quasiperiod intrinsically hard. But on the other hand, *aficionados* of QPO hunting would claim that the apparent correlation of frequency with intensity is just what one would expect.

There have been several claims of periods or quasiperiods from active galactic nuclei over the years, few of which have survived the test of time. Most notable is the never-ending saga of

the blazar OJ287, for which various periods between 15 and 44 minutes have been reported in these pages and elsewhere, but in summary judgement never convincingly demonstrated¹¹. So only with more data can we determine whether or not the QPO and its wanderings are persistent characteristics of NGC5548. □

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ASTRONOMY

Oldest stars are older still

Rosie Wyse

A LOWER limit to the age of the Universe can be obtained by dating the oldest objects in it; the oldest stars in our Milky Way Galaxy are then obvious targets for study. New observations reported by Lee¹ suggest that astronomers have been looking in the wrong place for these, and thus that they have underestimated the age of the oldest stars by 1–2 billion years. Not only would this alter our view of the Universe, but it would also affect our picture of the sequence of events that built our Galaxy.

Knowing where the oldest stars are in our Galaxy is the crux of the issue. This depends on how the Galaxy formed. The sites of the earliest star formation were probably the most dense regions of the protogalaxy, as intuition suggests that things happen faster when there is a crowd. The central region of the Galaxy, the nuclear bulge, has a stellar density orders of magnitude higher than that in the neighbourhood of the Sun, as do the populous globular clusters of stars in the halo. The stars in the globular clusters also have very low levels of the heavier elements produced in supernovae, their mean chemical abundance being one-thirtieth of that of the Sun; this is often an indicator that the stars formed early. And, indeed, these stars were thought until recently to be the oldest in the Galaxy.

In contrast, the stars in the nuclear bulge have on average twice the chem-

ical abundance of the Sun. So Lee's new analysis¹, suggesting that, nonetheless, the oldest stars of the Galaxy are to be found there, comes as a surprise. Lee proposes that the bulge stars are 1–2 billion years older than the previous record holders, the stars in the globular clusters, being perhaps 16 ± 2 billion years old. For cosmologists, this would provide another push in the direction of a non-zero cosmological constant (a kind of antigravity once described by Einstein) if the Universe has the critical mass for gravitational closure, as popularly supposed. Otherwise, the expansion age of the Universe, measured by the Hubble constant, which relates the velocity of recession of a galaxy to its distance from us, is in danger of being shorter than the age of the stars.

Lee's analysis, together with a related study by Suntzeff, Kinman and Kraft², indicates that there is an age gradient in the old stellar populations of the Galaxy, which would be consistent with the Galaxy forming from the inside-out. This is predicted in most theories of Galaxy formation when star formation rate is higher in regions of higher gas density, as galaxies may be expected to form with a smoothly decreasing mean density profile surrounding the centre. Thus a 'monolithic collapse' model which envisages a large, Galaxy-sized gas cloud collapsing under its own gravity, such as proposed by Eggen, Lynden-Bell and

Sandage³, will still be oldest in the central regions, where star formation may be expected to have been initiated first and proceeded fastest⁴. It remains to be shown that this picture is consistent with the relatively large amplitude of the age gradient inferred by Lee. A hierarchical clustering picture for the growth of galaxies and galaxy clusters, such as the still-popular cold-dark-matter cosmologies, envisages that the Milky Way formed by the gradual agglomeration of many smaller objects, perhaps with individual mass one-thousandth that of the present Galaxy. Such a 'chaotic' style of Galaxy formation was proposed by Searle and Zinn⁵ and is favoured by Lee. Again, only qualitative agreement with the inferred age gradient has been demonstrated. Indeed, both pictures have much in common⁶.

The main observation that Lee uses to infer relative ages involves the distribution in colour of stars in an advanced stage of evolution (the 'horizontal branch'), when the source of starlight is fusion of helium in the core, rather than the hydrogen fusion that powers the Sun. RR Lyrae variables, the particular type of star studied by Lee, form a subset of such stars, covering only a narrow range in colour, approximately in the middle of the observed range, which corresponds to the physical conditions that cause pulsational instability and variability. The chemical abundance with which a star is born affects its colour, and stars with too high a chemical abundance will be too red to become RR Lyrae variables. Some other parameter, most probably related to the mass of the star, also affects the colour distributions, as lower-mass stars have a bluer colour on the horizontal branch of globular clusters⁷. Stars of different masses evolve at very different rates, so that an older cluster has lower-mass stars reaching the horizontal branch, with a resultant bluer mean colour.

This effect allows an older star of high chemical abundance to be blue enough to be an RR Lyrae variable even though a younger star of the same chemical abundance would be too red to vary. And this is the crux of Lee's argument — the mean chemical abundance of the RR Lyrae variables in the nuclear bulge is higher than that of the RR Lyrae variables in other locations of the Galaxy, leading him to infer an older age for the bulge stars (but it is still only the low-chemical-abundance tail of the bulge stars that form RR Lyrae stars).

However, the mass of a star on the horizontal branch is also determined by the mass loss that occurs during an earlier phase of a star's life, when it is a cool, distended 'red giant'. Variable mass loss probably provides the dispersion in mass required for the observed

spread in horizontal-branch colour in a given cluster (where all the stars are the same age and chemical abundance)⁷. Indeed, at the high chemical abundances of red giant stars in the bulge, a difference of only 5 per cent in mass on the horizontal branch can move a star all the way from red to blue⁸.

There is, unfortunately, no good understanding of what physical parameters determine the mass loss rate. Observations suggest that a star with lower mass has a higher mass loss rate, and such a dependence reduces the required age difference between the nuclear bulge and halo by a third, as recognized by Lee⁹. It is plausible that chemical abundance is also significant here, with mass loss being higher for stars of higher chemical abundances, which would greatly complicate any age determination from the horizontal branch. This must be quantified before Lee's analysis can be treated as the final answer.

The very high chemical abundance of the bulge stars complicates essentially all other methods of dating the bulge. Study of faint, slowly burning, unevolved stars is more difficult, but both the state-of-the-art ground-based observations (S. Ortolani and M. Rich, personal communication) and Hubble Space Telescope data¹⁰ favour an age of perhaps 10 billion years for the dominant population. This highlights a further complication in the interpretation of any age determination as a constraint on Galaxy formation — tracers of all the stellar populations of the Galaxy are more numerous in the central regions, almost by definition. Stellar populations are essentially defined by age, chemical composition and kinematics (the shape of a typical orbit through the galaxy). Thus the ideal information would be the age distribution of stars of given chemical abundance distributions and kinematics. Such datasets will soon be possible, from a combination of surveys using multi-object spectrographs and advances in stellar evolution theory.

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The case of the velvet worm

Richard A. Fortey and Richard H. Thomas

It is often the case in zoology that the humblest animals are the most controversial. Those that are simplest in construction are either cited as primitive species which help to elucidate the major features in evolution, or are condemned as degenerate specialists, which have become secondarily simplified to occupy a *cul-de-sac* in the history of life. The velvet worms (Onychophora) are such a group of animals. They have long been

They feed by glueing their prey down with an adhesive which is shot from slime glands on the modified head of the animal. Such unpleasant habits are surely a secondary specialization, but in other ways onychophorans are like arthropods. They grow by moulting (ecdysis), for example, and their heart, antennae and brain have all been compared with the same organs in living arthropods. However, their method of



Robert Taylor

Euperipatoides kuczarti, an onychophoran — is it an arthropod?

supposed to be a kind of 'missing link' bridging the gap between annelids — segmented worms — and arthropods, that great group of jointed-legged animals that includes everything from spiders, scorpions, millipedes and mosquitoes in terrestrial habitats, to lobsters and extinct trilobites in the sea. Now a molecular study¹ reported in *Science* suggests that these extraordinary animals do not after all provide a bridge between phyla, but rather can be placed firmly within the arthropods. They then become another case of specialization imitating simplicity.

Onychophorans somewhat resemble caterpillars (see picture); most would fit comfortably into the palm of a hand. They have simple, rather stumpy, lobe-pod walking legs, which are quite different in their musculature from those of living arthropods. They do not have a thick exoskeleton, and they tend to be found in humid places, seeking cover in crevices or beneath rotting logs in dry weather. Like other evolutionary enigmas, they are probably more diverse in the Southern Hemisphere, but the best known of these obscure animals, *Peripatus*, has a circumtropical distribution.

locomotion, their poorly developed head, and their lack of an arthropod-like exoskeleton, have all suggested that the velvet worms are the more primitive. If so, understanding their relationships would be crucial to unravelling the origins of our most diverse group of living animals.

The fossil record supports this notion. *Aysheaia* is a fossil animal from the famous Middle Cambrian Burgess Shale², which was almost certainly a marine lobopod. Other peculiar fossils that have been claimed as "unknown phyla"³ have been reinterpreted as early onychophorans⁴; thus it seems likely that they were more diverse in the Cambrian than they are now, and radiated early along with other major arthropod groups⁵ in the marine environment. Like the arachnids (spiders and scorpions) and the myriapods (centipedes and allies), the move to terrestrial environments happened later.

The doyenne of arthropod specialists was Sidonie Manton, who used morphological evidence to conclude that onychophorans were related to myriapods and hexapods (insects and allies)⁶, a group typified by having unbranched limbs

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(Uniramia). Others preferred to regard the Onychophora as a primitive sister group to the arthropods as a whole; insects may have had crustacean-like ancestors⁷, a supposition supported by the similarities in the compound eyes of the two groups. In these very different scenarios the place of the Onychophora is clearly pivotal. But as their morphology and embryology do not supply an unequivocal way of deciding between such conflicting theories (and there are several more), new and critical evidence is needed. The insights provided from sequencing pieces of DNA from different organisms and comparing and ordering the changes in evolutionary sequence ought to provide just such a key. Recent studies on sequences of 18S ribosomal RNA genes⁸, for example, have demonstrated that the arthropods as a whole were very probably descended from a common arthropod-like ancestor.

This is where the results of Ballard and his co-workers¹ are so surprising. On the basis of sequences of a 329-base pair piece of the mitochondrial 12S ribosomal RNA gene from a wide range of arthropods, together with six onychophoran species, and a range of organisms from other phyla, the Onychophora are resolved in a position within the arthropods, which implies that their apparently simple structure is secondary. If so, the poorly defined head region of the velvet worms would have to be a degenerate feature, which might be considered as a response to their specialized life style. Possibly even more unexpected is the discovery that the myriapods are identified as the sister group of all other arthropods, because these arthropods had not previously been allotted such a fundamental role. In any case, both onychophorans and myriapods were separated from hexapods, and thus Manton's Uniramia has been thoroughly dismembered.

These results are a tribute to molecular studies as a stimulus to new ideas. But before we can allow *Peripatus* and its friends to creep away under a mossy log into an evolutionary backwater, it is worth recalling that these molecular data are still imperfect, and that confirmation is needed from other sequence data from different genes. The divergence of the various arthropods happened a vastly long time ago, making it difficult to discriminate the signals of these distant

evolutionary events from the 'noise' of 500 Myr of subsequent evolution. Our own analysis of Ballard *et al.*'s original data indicates that the base changes supporting the phylogeny, particularly the placement of the myriapods, are few in number and questionably robust. However, Ballard *et al.* have been very clear in the presentation of their methods and assumptions, which will help in any critical evaluation of onychophoran relationships in future. At

present, the fossil record, morphology and molecules all point to different conclusions, but none of them is without flaws. A more definitive answer may come from discovering the right genes to unscramble the inscrutable past, but at the moment the role of the velvet worm in the history of life is still not proven. □

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TUMOUR NECROSIS FACTOR

Improving on the formula

Frances Balkwill

Is tumour necrosis factor (TNF) appropriately named? Early on it seemed that it was¹. The gene for this multifunctional cytokine was cloned in 1984, and the recombinant molecule had tumour necrotic activity² and was cytotoxic for tumour cells when combined with interferon- γ (ref. 3). But the variety of TNF action on a wide range of cells, and its severe toxicity in early clinical trials, soon dampened optimism that it could be used in the treatment of cancer. There was also strong evidence that dysregulated production of TNF was involved in the pathophysiology of several infectious and autoimmune conditions, most notably endotoxic shock⁴ and rheumatoid arthritis⁵. One way round these drawbacks would be to engineer a molecule with some of the anti-cancer properties of TNF but without its toxicity, and that is what Van Ostade *et al.* may now have achieved (see page 266 of this issue⁶).

The authors have exploited the fact that TNF binds to two distinct cell-surface receptors (TNF-R55 and TNF-R75) which may mediate different biological actions⁷. The first of these receptors is expressed on a wide range of cells and is sufficient to trigger the cytotoxic action of TNF on tumour cells, whereas the second shows a more restricted expression, primarily on lymphoid or myeloid cells. There is some degree of species specificity in the binding of TNF to its receptors; human TNF binds only to murine TNF-R55, but both human and murine TNF bind with equal affinity to human TNF-R55 and R-75 (ref. 8). Human TNF is 50 times less toxic than murine TNF when administered to mice, implying that the murine TNF-R75 mediates systemic toxicity.

Van Ostade *et al.* have characterized a human TNF mutant (a change of arginine to tryptophan at position 32) that has a 600-fold lower affinity for human TNF-R75 but retains its binding properties to human TNF-R55. This mutant

TUMOUR NECROSIS FACTOR: THE THERAPEUTIC PROS AND CONS

Pros

Cytostatic/cytotoxic action on tumour cells
Destruction of tumour vasculature
Stimulation of host immunity

Cons

Autocrine/paracrine stimulation of tumour cell growth
Increased tumour cell motility
Stimulation of angiogenesis
Tissue remodelling to enhance invasiveness
Contribution to cachexia (wasting)?

cytokine slows down the growth of a human tumour-cell line growing subcutaneously in nude mice and, in combination with low doses of human interferon- γ , causes a strong, but reversible, abrogation of tumour-cell growth. The mutant TNF thus has potential as an agent that will inhibit tumour-cell growth in the presence of other cytokines or chemotherapies with which TNF has shown strong synergy. Preclinical data from other centres¹, and the experiments reported in the current paper, suggest that it would be less active on its own.

However, as the authors of the paper point out, in many animal tumour models and in clinical trials where the cytokine is administered loco-regionally⁹, the anti-tumour activity of TNF involves other actions. The apparent selective action of TNF on the neovasculature in the tumour microenvironment, and the stimulation of T-cell-mediated immunity to tumour cells, may be of crucial importance. It is likely that TNF-R75 is involved in mediating some of these actions. Another important qualification is that the requirement for TNF-R55 expression for TNF's cytotoxic effect may not be absolute — in some cell lines TNF-R75 is necessary and sufficient for cytotoxic action¹⁰. Moreover, human TNF can be toxic in mice that are also given sensitizing agents such as interleukin-1 β , lipopolysaccharide or a glucocorticoid antagonist.

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Only when the TNF mutant described by Van Ostade *et al.* enters toxicity trials in primates or is used in clinical cancer trials will we know whether the approach has been successful. Even if toxicity is reduced and anti-cancer activity evident, it will be necessary to show that this advantage is not offset by increased antigenicity of the mutant molecule, or expression of some of the potential tumour-promoting actions of TNF.

All these arguments make the assumption that TNF treatment would be good for the patients and bad for the tumour. But there is increasing evidence that dysregulated cytokine production may be involved in the malignant process, as it is in infectious and autoimmune disease (for review see ref. 1). The response of a tumour to a cytokine will depend on the context in which the signal is received — sustained local levels of TNF or other soluble mediators, the state of tissue differentiation and the phase of the cell cycle will all influence the response. In human ovarian cancer TNF is implicated as a paracrine/autocrine factor that may promote tumour growth and spread¹¹, and TNF treatment of human ovarian cancer xenografts growing in nude mice stimulates autocrine production of TNF by the tumour cells (unpublished data of my own group). Several actions of TNF, for example stimulation of protease activity and angiogenesis, would make it a useful secretory product for a metastasizing tumour. The balance between these positive and negative actions of TNF in malignant disease is shown in the table.

The clinical promise of other cytokines, most notably interleukin-2 (IL-2), is also limited by their toxicity. Indeed, the systemic induction of TNF during IL-2 therapy has been implicated in side effects such as hypotension, fever, rigors and weight gain. There are no data supporting the idea that a mutant IL-2 molecule might have an increased therapeutic index, and although the IL-2 receptor complex has at least three compo-

nents it does not seem that they regulate different actions. Animal tumour experiments, in which TNF production is suppressed during IL-2 therapy, give conflicting results¹², but it is possible that toxicity can be reduced without affecting IL-2's anti-cancer activity¹³.

However that may be, the paper by Van Ostade *et al.* raises important issues. If the complexity and toxicity of

cytokine action can be restricted by the use of mutant molecules or specific inhibitors, then the therapeutic potential of these mediators may be greatly increased. □

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CHEMISTRY

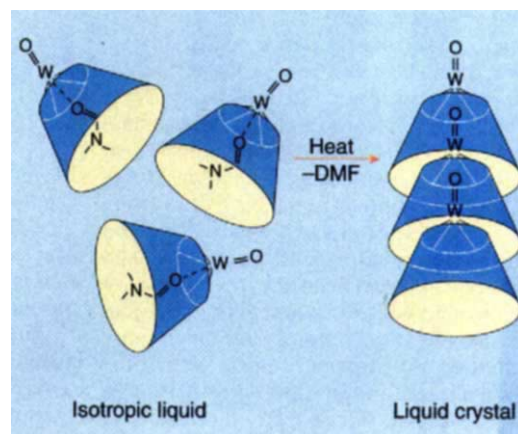
Nanometre assembly lines

Thomas Bein

CHEMISTS and materials scientists aspire to the design and investigation of ever more complex molecular structures and assemblies, which could be used in subtle optical technologies, as ferroelectric (spontaneously polarized) materials, sensors, computer memory elements and catalysts, and in other applications mimicking biological functions. Complex molecules and supramolecular associations between individual molecules are succumbing to this onslaught, but the control of molecules at the larger scale of assembly is much less developed: one can crystallize sugar from water, but with little control over the precise superstructure (crystal) eventually assembled. However, *en route* to complete supramolecular control, exciting progress has been made in the synthesis of functional thin films, ordered liquids and polymers, large clusters and complex host-guest systems, as was apparent at a meeting of the Materials Research Society last month*. Examples of linear, two-dimensional layer and three-dimensional cluster assemblies demonstrate the power of novel synthetic strategies.

To show ferroelectric or second-order nonlinear optical behaviour (such as intensity-dependent refractive index), a system cannot have a centre of symmetry. An approach now being developed is to use the properties of liquid crystals to align large arrays of such molecules for practical purposes. T. M. Swager (University of Pennsylvania) described liquid crystals based on bowl-shaped tungsten-oxo calix[4]arene complexes in which the tungsten-oxygen groups serve as dipoles and stimulate head-to-tail organization in columnar (discotic) phases of unusually high stability. Discotic phases look

like tightly packed stacks of coins. The azo-substituted precursor calix[4]arenes do not form stable liquid crystals, but when the four hydroxyl groups at the rim of the calixarene bowl are capped with the tungsten-oxygen group (see figure)



Dimethyl formamide (DMF: $\text{O}=\text{CH}-\text{N}-(\text{CH}_3)_2$) blocks the head-to-tail formation of the calixarene mesophase by binding inside the larger molecule's bowl-shaped cavity.

by reacting them with WOCl_4 , discotic columnar liquid-crystal phases are formed that are stable over a spectacular range of about 200 °C. The source of the stability may be indicated by experiments with Lewis acids, which become bound into the molecular cavities. Thus dimethyl formamide (DMF) forms strong complexes that are detected by changes in the $\text{W}=\text{O}$ frequencies and NMR data. The DMF guest reversibly suppresses the liquid-crystal formation, suggesting that the tungsten-oxo calix[4]arene complexes (without DMF) organize in head-to-tail structures where the $\text{W}=\text{O}$ group of one molecule protrudes into the calixarene cavity of the next molecule (see figure). Although the ferroelectric and optical properties of this system have yet to be explored, Swager is encouraged by results from a simpler liquid-crystal system based on vanadium-oxo head-to-tail linear-chain compounds, which are ferroelectric. The

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spectacular stability of the tungsten-oxo liquid crystals should make these suitable for both ferroelectric and nonlinear optical applications.

Even rain can benefit from the design of supramolecular assemblies, particularly when they are in the form of two-dimensional networks. In many areas of our planet one would like to seed, or crystallize, the water droplets of clouds residing in the sky at temperatures in the range 0 to -8°C . Highly effective nucleation promoters for ice are now being constructed where structural relationships exist between an amphiphilic assembly and the ice surface (M. Lahav, Weizmann Institute of Science, Rehovot). For example, amphiphilic alcohol molecules $\text{C}_n\text{H}_{2n+1}\text{OH}$ which aggregate at the water-air interface expose a network of hydrophilic head groups which can complement a layer of the nucleating ice crystal.

A correlation was previously observed between the freezing point of super-cooled water and the alcohol chain length. Grazing-incidence X-ray diffraction using synchrotron radiation shows that uncompressed alcohol layers with 23, 30 and 31 carbon atoms in the chain have a distorted hexagonal unit cell of side 4.5 Å, very similar to that of the (0001) face of hexagonal ice (also of side 4.5 Å). Recent electron microscopy and diffraction studies confirm the epitaxial arrangement between the alcohol monolayers and hexagonal ice. Shorter alcohol chains (16 and 20 atoms) are much more tilted on the air/water interface than the longer ones, and so have unit-cell dimensions very different from those of ice. This can explain why longer alcohol chains nucleate ice at higher temperatures than do shorter chains.

Surprisingly, alcohols with odd-numbered carbon chains are much better nucleators than those with even-numbered chains (with a 6–7 $^{\circ}\text{C}$ difference in freezing point), even though the unit cells are very similar. By using alcohols bearing ester or amide groups in the middle of the chain with odd or even number chains reaching down into the water, Lahav and colleagues have shown that the orientation of the hydroxyl groups also plays a critical role in nucleation. An odd-numbered alcohol chain with 31 carbon atoms can nucleate ice at around -0.5°C , a great achievement compared with the -14°C achieved with a 16-carbon alcohol. These and related strategies are now being explored for the seeding of clouds and for oriented crystallization of other substances using amphiphilic networks.

Tiny semiconductor clusters have long attracted the attention of solid-state scientists who are fascinated by the dramatic changes of electronic and optical properties when reducing the size of

these materials from bulk to nanometer dimensions. One potential application of these so-called quantum dots is in optical transistors, where light intensity can be controlled with light. To date, preparations of these species have lacked the precise control in size necessary for the optimum physical properties.

A cluster approaching the holy grail of this area has now been synthesized by N. Herron and colleagues at DuPont Central Research. $\text{Cd}_{32}\text{S}_{14}(\text{SPh})_{36}(\text{DMF})_4$, the largest metal chalcogenide cluster ever to be characterized by X-ray crystallography, is formed by controlled pyrolysis of a smaller cluster anion, $[\text{Cd}_{10}\text{S}_4(\text{SPh})_{16}]^{4-}$, as an intermediate during the transformation to bulk CdS. The trick of this fusion is to distil four of the thiophenolate capping ligands from the precursor cluster together with the four cations NMe_4^+ as MeSPh and NMe_3 . The 82-atom core of the resulting

cadmium-sulphide cluster is a 12-Å piece of the sphalerite lattice (one of the structures of bulk CdS) with reduced Cd-S bond length, capped with wurtzite-like CdS units. The tetrahedral clusters then pack themselves in a very open structure with 8-Å channels and 16-Å cavities that can adsorb solvents such as pyridine. Solutions of the Cd_{32} clusters exhibit strong and sharp exciton absorption at a wavelength of 358 nm, as predicted for a quantum dot of this size. Third-order nonlinear optical behaviour and photoconductivity of this material are now being explored. Indeed, the DuPont workers have already applied for a patent for using the clusters as a novel photoconductive component in photocopiers. □

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CLIMATE

Northward march of spruce

John Pastor

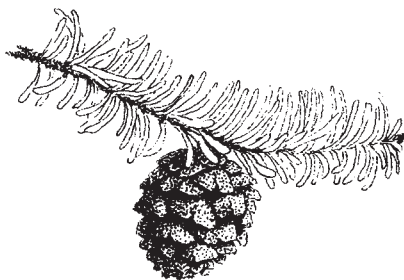
How quickly can ecological communities change as the climate warms? Very quickly at the forest-tundra border, say MacDonald *et al.* in a study of vegetation in Canada over the past 1,000 years, on page 243 of this issue¹.

With fine-scale resolution of pollen assemblages in cores collected from several lakes in central Canada, anchored by ^{14}C dates, MacDonald *et al.* show that the changes from tundra to closed-canopy black-spruce (*Picea mariana*) forest occurred over a 150-year interval between 5,000 and 4,000 years ago. The invasion was forced possibly by a regional northward shift in the summer position of the Arctic front. Diatom assemblages in the same cores show that the lake community shifted at exactly the same time from an acidophilous

low-productivity community to a more productive one, indicative of higher pH levels. Lagging slightly behind these terrestrial and aquatic community changes were increases in total lake productivity and watershed runoff, and decreases in evapotranspiration, as indicated by changes in isotope ratios of the diatoms and lake carbonates.

Climate changes can theoretically force rapid changes in ecosystems if temperature thresholds favouring one or more species are crossed and if strong feedbacks operate between the favoured species and the environmental conditions that determine their dispersal, establishment and growth^{2,3}. These feedbacks can occur with population, ecosystem or landscape processes and can amplify the effects of gradual or even small changes in climate. Presumably, the more levels of ecological organization there are having thresholds and feedbacks with invading species, the greater the potential for sudden change once thresholds are crossed. The rapid changes found by MacDonald *et al.* in terrestrial and aquatic communities, nutrient cycles and watersheds are consistent with such catastrophic behaviour, which has rarely been demonstrated empirically.

What are the sequences of events and ecological processes underlying this invasion of tundra by black-spruce forest? Spruce has a particularly interesting pattern in the fossil pollen record in that it appears⁴ to have migrated north at approximately 200 km per century since



Cone and twig of the black spruce, *Picea mariana*, whose traits facilitate the species' invasion of tundra. Fires promoted by the inflammable needles open the serotinous cones, so encouraging the spread of the seeds to the detriment of stunted krummholz forms, which spread by vegetative layering.

the last ice age ended approximately 9,000 years ago. That migration is almost an order of magnitude faster than for other tree species of eastern North America⁵. Black spruce, in particular, has a number of interesting characteristics in its life history that may facilitate rapid invasion of tundra once climatic thresholds are crossed.

Winter temperature is an important threshold that, once crossed, can trigger phenotypic changes in form and dispersal mechanisms within populations. Spruce at the treeline can assume one of two forms: stunted individuals (krummholz) that regenerate vegetatively by layering, or upright trees that regenerate mainly by seed dispersal⁶. The krummholz form dominates when winter air temperatures are very low, because the apical buds remain below the surface of the snow and are not killed by frost. With milder winter conditions, however, apical buds can survive above the snow–air interface and upright trees are favoured. These trees disperse seed during subsequent summers, thereby providing foci for rapid invasion once the winter threshold is crossed⁶. Such an invasion sequence is indicated by MacDonald *et al.*'s pollen record, which shows sporadic and low abundance of spruce pollen before 5,500 years ago with rapid increases thereafter.

It is possible that spruce invasion also alters the ecosystem in its favour, for instance by changing the cycling of limiting nutrients between plants and soils^{3,7}. Spruce litter decays and releases nutrients faster than that of lichens in the tundra, for example⁸; this enhancement of nutrient cycles may support the higher productivity and canopy closure of boreal forests, thereby shading out the low-statured tundra species.

At the landscape scale, there is a feedback between spruce invasion and fire regimes⁹. Some individuals of black spruce have serotinous cones, that is, they do not shed seed until they are opened by fire. With its high resin content, black-spruce litter is highly inflammable, thereby creating a fire regime conducive to its dispersal. Both fire frequency and size increase markedly south of the boundary between

tundra and boreal forest, perhaps because of the association between litter inflammability and serotiny in black spruce⁹. Changes in fire regimes elsewhere in Canada have triggered rapid and major changes in forest composition¹⁰, especially with regard to serotinous and inflammable species such as black spruce and jack pine (*Pinus banksiana*). Although MacDonald *et al.* do not report any data on charcoal abundance, such changes in fire regimes with climate warming cannot be ruled out for the region.

It would be interesting to determine if catastrophic changes are symmetrical at the forest–tundra border. That is, can tundra reinvade boreal forest as rapidly with climatic cooling? It would seem not, as it would require wholesale opening of the forest canopy for tundra species to

invade and no tundra species has the full suite of characteristics necessary to facilitate rapid invasion, whereas spruce does.

The timescale of the changes found by MacDonald *et al.* agree with those predicted by model simulations of regional response to anticipated warming from the greenhouse effect^{3,8}, and they offer the prospect of further advances in treeline. The challenge now is to combine regional circulation models of the atmosphere with vegetation models that incorporate the coupling of species life-histories to ecosystem and landscape processes. Only then will it be possible to get a complete picture of regional responses to global climate change. □

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PHYTOPLANKTON

Limits on growth rates

J. A. Raven

At first glance, carbon would seem to be the last nutrient to be rate-limiting to the growth of photosynthetic plankton in the oceans, as the concentration of dissolved inorganic carbon in sea water is much greater than that of other essential nutrients such as nitrogen. The atmospheric concentration of carbon dioxide has therefore not generally been considered to have any effect on the biological carbon 'pump' in the oceans. But from the results of computer modelling and experiments reported on page 249 of this issue¹, Riebesell *et al.* suggest that the supply of CO₂ for photosynthesis can indeed restrict the rate of new primary production in plankton.

The role of the oceans as a sink for atmospheric CO₂ is currently of great interest because of its effects on the increase of CO₂ in the atmosphere and, by implication, on forecasts of likely global warming. Net removal of CO₂ from the atmosphere is due both to purely chemical and to biological processes. Biological removal is mediated by the sedimentation of 'new production' — the biomass generated by photosynthetic microorganisms — which takes the fixed carbon down to the depths of the ocean.

The extra nutrients, such as nitrogen and phosphorus, that are needed for new production (that is, extra to those recycled by decomposition through the upper levels of the ocean) come from upwelling from the deep ocean². But how can CO₂ be limiting? One part of the answer lies in the fact that more than 95 per cent of carbon fixation in phytoplankton is by the photosynthetic carbon-fixation reaction mediated by the

enzyme RUBISCO (ribulose biphosphate carboxylase oxygenase), which can use only CO₂. CO₂ is also the substrate for the carboxylase (phosphoenolpyruvate carboxylase) responsible for most of the remaining C fixation by diatoms. The bulk of dissolved inorganic carbon in the oceans is, however, in the form of HCO₃⁻ (ref. 3). So one possible kinetic limitation on the use of the apparent excess inorganic carbon is the rate at which HCO₃⁻ is converted to CO₂.

Biological membranes are intrinsically highly permeable to gaseous CO₂ and so the obvious route for inorganic C entry is by CO₂ diffusion across the plasma-membrane and plastid-envelope membranes as in terrestrial C3 plants. But aquatic plants have a constraint on CO₂ supply to the outer surface of the plasma-membrane. This relates to the lower diffusion coefficient of CO₂ in water than in air, which is not offset by the thinner diffusion boundary layer around a phytoplankton cell in water than that around a leaf in air. This constraint is partially offset by conversion of HCO₃⁻ to CO₂ within diffusion range of the plasma membrane^{4,5}.

Riebesell *et al.*¹ modelled the diffusive CO₂ flux, including the contribution of the slow (when uncatalysed) conversion of HCO₃⁻ to CO₂, to the surface of a diatom cell, and compared it with the computed fluxes of NO₃⁻ and HPO₄²⁻ from bulk-phase sea water with a CO₂:NO₃:HPO₄ ratio approximating to that in an upwelling bloom or in a high-latitude spring bloom. When they set the steady-state concentration at the cell

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surface to the same fraction of the bulk-phase concentration for all three solutes, the computed ratio of the rates of C:N:P supply to the cell surface of 28:38:1 deviates from the ratio of 106:16:1 needed for phytoplankton growth (the Redfield value) in a way that suggests C limitation. When they set the CO_2 and NO_3^- fluxes to the cell surface to a Redfield ratio of 106:16 for a given specific growth rate, they found that the steady-state concentration difference between bulk sea water and the cell surface is much higher for CO_2 than for NO_3^- , both in absolute terms and as a fraction of the bulk seawater concentrations. This again suggests that C limits growth to a greater extent than does N.

The modelling was paralleled by the study of laboratory cultures of three species of oceanic diatoms. Their results suggest that any decrease in free CO_2 concentration, as a result, in bloom conditions, of CO_2 fixation by RUBISCO faster than CO_2 is resupplied from the atmosphere, would reduce the rate of primary production. Riebesell *et al.* also found that the rate of inorganic C uptake was no greater than could be accounted for by diffusion of CO_2 and uncatalysed conversion of HCO_3^- to CO_2 .

The inorganic C utilization by these three diatoms can thus be explained by diffusive supply of CO_2 to the cell surface without extracellular catalysis of the HCO_3^- to CO_2 conversion, though a direct inhibition of a mechanism for HCO_3^- use by high pH cannot be ruled out.

The mechanism by which CO_2 enters the cells remains open to question. The high affinity (low K_s) for inorganic C, expressed as the K_s for free CO_2 of 1.2–2.1 mmol CO_2 , suggests active CO_2 influx, as the $K_{1/2}$ for CO_2 of diatom RUBISCOs is an order of magnitude larger than these K_s values. While the K_s could be explained by diffusive entry of CO_2 and the presence of CO_2 -saturated RUBISCO activity at least ten times greater than the inorganic C-saturated photosynthetic rate *in vivo*, such excess RUBISCO contents in rapidly growing microbial phototrophs are unprecedented⁶. By contrast, active CO_2 influx, leading to a higher CO_2 concentration around RUBISCO than occurs just outside the plasmalemma in steady-state photosynthesis, is known to occur in photosynthetic microorganisms^{4,5}.

A further twist to the inorganic speciation story relates to the ability of some photosynthetic microalgae to use HCO_3^- (where HCO_3^- use is defined as photosynthetic inorganic C assimilation in excess of what can be explained by uncatalysed extracellular conversion of HCO_3^- to CO_2 with subsequent CO_2 uptake^{2,4}). HCO_3^- use may involve extracellular catalysis of HCO_3^- to CO_2 conversion with subsequent CO_2 uptake,

or active HCO_3^- influx. HCO_3^- use is widespread in photosynthetic microalgae, although its extent, like that of CO_2 active transport, may have been overestimated for marine photosynthetic microorganisms growing in air-equilibrated sea water. The long-term nature of the experiments of Riebesell *et al.* means that any such acclimation is incorporated into the data at low inorganic C concentrations, so that their results provide a true reflection of growth responses to a range of inorganic C concentrations. It may, therefore, be more than coincidental that the K_s (CO_2) values for the three diatoms² are between the $K_{1/2}$ (CO_2) values for photosynthesis in air-acclimated and in low inorganic C-acclimated diatom cultures⁷.

Regardless of the details of inorganic C uptake, however, the results obtained by Riebesell *et al.* are consistent with inorganic C limitation of growth rate in 'new production' conditions in the ocean. They are at pains to point out that the inorganic C limitation concerns rate rather than extent. Accordingly, any rate-limitation of new production in the ocean by inorganic C is likely to result in an increased temporal (spring bloom) or spatial (coastal upwelling) extent of the new production. There would be no difference in the annual global new production relative to a world with no inorganic C limitation, provided that the loss terms (for example, as a result of phytoplankton sinking) are not functions of growth rate. Riebesell *et al.* do indeed suggest that losses due to sinking increase with growth rate, so it could be that as inorganic C, or more specifically CO_2 , concentrations in the ocean increase, so does organic C burial. This could be important for the interpretation of what was happening in glacial periods when atmospheric CO_2 partial pressure was only half what it is today. Furthermore, recent work suggests that there has in fact been no increase in the biomass of North Pacific phytoplankton in the past 90 years of atmospheric CO_2 increase⁸, a finding consistent with, but not constituting a rigorous test of, the thesis of Riebesell *et al.* □

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Healthy building

MODERN architecture has given us many quite deliberate horrors. It has also created an accidental one, the 'sick-building syndrome'. This arises from the misguided attempt to reduce heat losses and to control the internal environment by sealing all openings to the outside air. The stagnant atmosphere in the building rapidly accumulates solvent vapours from plastic surfaces, ozone from the electrics, bacteria and biological organics from the occupants, and particles from everything. The staff, unable even to open the windows, feel dreadful. They also face the sinister threat of radon, which is emitted from granite bedrock or stonework and builds up relentlessly within the hermetically sealed structure.

Daedalus now suggests a solution. He claims that a porous wall, with air being sucked steadily inwards through it, must be a perfect heat insulator. As fast as heat leaks outwards by conduction, it is collected and returned by the inward flow of air. Good ventilation is thus combined with perfect heat retention. To prevent rising damp and penetrating rainwater, Daedalus proposes to treat the masonry with organosilane halides, which attach water-repellent hydrocarbon chains to the hydroxyl sites on the brickwork. Like porous PTFE fabric, the resulting structure will be totally waterproof but easily permeable to air. A building constructed in this manner could inhale continuously through its whole surface.

This elegant construction will combine free ventilation with almost perfect heat retention. Heat will escape only through the ventilation exhaust which exhales the warmed inspired air; but the thermal capacity of air is so low that heating bills should still plummet. The happy occupants will be bathed in fresh, warmed, filtered air, emerging gently and evenly from walls, floor and ceiling. The interior decoration will stay bright and sparkling, for no dust will settle on it. The upflow through the porous floor may cause carpets to drift uncannily around unless tacked firmly down; smooth-soled shoes may slide awkwardly on the slippery air-bearing surface, while cats and dogs could resent the ventral ventilation when they sit down. But the outside of the building would soon look hideous. It would rapidly suck onto itself dust, grime, leaves and scraps of paper and plastic sheeting. For some reason, such random, unplanned visual squalor offends the nightmare vision of the modern architect. Perhaps the pumps should be reversed for a short time each night, to blow the garbage off the structure and restore its naked monolithic ugliness. David Jones

Viscosity of the asthenosphere

SIR — Foulger *et al.*¹ have used the observation of transient strain following dyke intrusion in northeast Iceland to estimate a newtonian viscosity for the asthenosphere; their value ($\sim 10^{19}$ Pa s) is of the same order as that obtained from postglacial rebound². Observations such as those presented by Foulger *et al.* are very important, as they sample timescales (~ 1 – $1,000$ years) for which data are scarce and the rheological behaviour of the crust and mantle may not be steady-state. However, I argue here that the data do not provide a strong constraint on the viscosity of the asthenosphere, because (1) the viscous layers in stress relaxation and in postglacial rebound are not necessarily the same; (2) the estimated viscosity depends on the kinematics of flow; and (3) the inelastic response over ~ 10 yr is likely to be transient and not steady-state.

The model of Foulger *et al.* assumes an elastic layer of thickness $h = 8$ – 30 km and modulus $M = 0.75 \times 10^{11}$ Pa overlying a viscous layer of thickness $b = 5$ – 10 km, identified with a layer of partial melt. A boundary displacement then propagates in the elastic layer according to the diffusion equation³. However, postglacial flow affects a sub-lithospheric layer at least one order of magnitude thicker⁴, which, using the inferred diffusivity, would give a correspondingly higher viscosity. Furthermore, the diffusivity used in ref. 1 ($\kappa = Mhb/\eta$) is valid only for a linear velocity–depth distribution. If the distribution is quadratic (return flow within the viscous layer) the diffusivity is⁵

$$\kappa = \frac{Mhb^2}{2\eta(3h+2b)}$$

which, for the same parameter values as in the linear case, gives $\eta \approx 10^{17}$ – 10^{18} Pa s, and $\eta \approx 10^{19}$ Pa s if $b \approx 100$ km. A different kinematics of flow, therefore, makes a thick channel compatible with postglacial viscosity estimates.

Finally, as the Maxwell time of rocks is $\geq 1,000$ yr, stress relaxation at plate boundaries, with a characteristic time of a few years, is unlikely to sample the steady-state rheology of the asthenosphere⁴. The transient strain rate of rocks at high temperature T usually depends on time t and stress σ as^{4,5}

$$\dot{\epsilon} = C\sigma^n t^{m-1} \exp(-E/RT)$$

where R is the gas constant, and the material parameters for silicate rocks are^{5,6} $C \approx 10^{-4}$ Pa ^{$-n$} s ^{$-m$} , $n \approx 2.0 \pm 0.5$, $m \approx 0.4 \pm 0.1$ and $E \approx 400 \pm 150$ kJ mol ^{-1} . The transient viscosity $\eta(t) = \sigma/\dot{\epsilon}$ can be expressed in terms of strain rate,

whose upper limit can be taken as $\dot{\epsilon} = \epsilon/t \approx 8.6 \times 10^{-15}$ s ^{-1} , where $\epsilon \approx 15$ cm per 50 km is the observed strain (one would obtain $\dot{\epsilon} \approx 5.2 \times 10^{-15}$ s ^{-1} assuming validity of the diffusion equation). For $t = 11$ yr (time of observation of the Iceland transient), $T = 1,500$ K, and central values of the material parameters, this yields $\eta \approx 4 \times 10^{18}$ Pa s, that is, not significantly different than in the linear model.

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Mimicking ligands

SIR — S. Davis *et al.* (*Nature* **358**, 76–79; 1992) explore the interaction of the HIV-1 glycoprotein gp120 and anti-CD4 antibodies with the CD4 receptor. They have carefully documented the binding characteristics of 225 anti-CD4 antibodies, and conclude that none represents an “exact mimic” of the binding of gp120. They further suggest that the underlying concept behind the use of an antibody to mimic a naturally occurring ligand is inherently flawed and “unlikely to be rewarding.”

One of their primary arguments against these antibodies appropriately mimicking gp120 is a significant difference in CD4 binding kinetics — k_{on} and k_{off} rates — between the antibodies and gp120. They therefore conclude that “interactions of antibodies and gp120 with CD4 may involve different types of binding sites or that regions outside the binding site affect the k_{on} .” They further argue that potential variability in antibody binding sites is so large as to make the use of anti-idiotypic antibodies as probes for a receptor essentially fruitless.

We believe that the binding of many agonists and antagonists to well-characterized receptors fails to satisfy the criterion of equivalent binding kinetics. A case in point is one of the best understood receptors, the nicotinic acetylcholine receptor. Nicotine, carbachol, tubocurarine, α -bungarotoxin, maleimido-benzyl-trimethyl-ammonium

and acetylcholine are agonists or competitive antagonists for the nicotinic receptor. They exhibit mutual competition, and furthermore have been of great utility in unravelling the structure and function of the nicotinic receptor. The structures of these agonists and competitive antagonists vary widely. If presented with these molecules *in vacuo*, it is unlikely that one would conclude that they bind in any biologically significant way to the same receptor. Anti-idiotypic antibodies — which may bear little obvious resemblance in either chemical structure or binding kinetics with the naturally occurring ligand — might also prove informative in the characterization of novel receptors.

Although we concur with the authors’ stringent criterion for the complete elucidation of a novel receptor, we would suggest that intermediate steps in the process might also be of general interest. We submit that the difficulty in the use of antibodies in unravelling the CD4/gp120 interaction is insufficient grounds for a complete indictment of either the technique or of results obtained with anti-idiotypic antibodies.

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SIR — On the basis of a study of one panel of antibodies specific for a ligand of one receptor, a limited review of the literature and a highly restricted criterion for a molecular mimic, Davis *et al.*¹ conclude that their experiments “undermine the concept of mimicry between antibodies and receptors”. They go on to state that the “anti-idiotypic approach [to the study of receptors] is unlikely to be rewarding”. We disagree. Our experience has shown that the immune repertoire is large enough to yield suitable mimics if proper, rigorous screening procedures are used to find them.

Molecular mimicry by an antibody must be defined operationally. Let us take as one example a monoclonal anti-idiotypic antibody that mimics aldosterone, which was isolated in my laboratory using an auto-anti-idiotypic strategy^{2,3}. It has the following characteristics: (1) It binds to Fab fragments of rabbit anti-aldosterone antibody with an affinity of 0.5 nM. (2) It inhibits the binding of [³H] aldosterone to rabbit polyclonal antibodies and to seven different aldosterone-specific monoclonal antibodies. (3) It inhibits the binding of [³H] aldosterone to rabbit kidney cytosolic aldosterone receptor preparations but does not bind to glucocorticoid

receptors. (4) It displaces [^3H] aldosterone from native 9S receptor as determined by glycerol gradient centrifugation. (5) Displacement of [^3H] aldosterone from the receptor has the same kinetics as unlabelled aldosterone. (6) It can detect mineralocorticoid receptor in kidney tissue by immunocytochemical procedures and is displaced by aldosterone. (7) It can detect aldosterone receptors in COS cells transiently expressing transfected cDNA that coded for the receptor.

Is this antibody a mimic of aldosterone? We think it is, even though it does not meet the "strict" (but dubious) criterion of Davis *et al.* that requires it to be used to discover a new receptor. There are other examples as well⁴.

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DAVIS REPLIES — Heng and Dreyer propose that anti-idiotypic antibodies need not be structural mimics to be useful for the characterization of new receptors. They cite the example of the nicotinic acetylcholine receptor, for which there exist several structurally unrelated ligands. We would not dispute that such molecules may be useful for characterizing a receptor. However, these molecules are functional — rather than structural — mimics and the anti-idiotypic approach to receptor characterization depends crucially on the belief that antibodies carry the "internal image" of the immunizing ligand and that anti-idiotypic antibodies are, potentially, structural mimics of the ligand¹. Considerable effort has gone into attempts to show², albeit unsuccessfully in our view, that for some model systems anti-idiotypic antibodies share significant structural homology with the relevant protein ligand. If the objective of the approach was not to obtain structural mimics then there seems little point in starting by immunizing with the ligand. Heng and Dreyer conclude by suggesting that data from the intermediate stages of experimental work before the complete elucidation of a novel receptor may be valuable. Although we totally concur with this view it will only be true if the data are relevant to the receptor under study. In the light of our results we are forced to conclude that this is unlikely to be the case in most, if not all, instances involving the anti-idiotypic approach.

Erlanger disputes our definition³ of a useful anti-idiotypic antibody and reiter-

ates the operational criteria, most of which have been in use since the inception⁴ of the anti-idiotypic approach to identifying receptors. In doing so, however, he fails to confront the key findings of our paper³. Regardless of their nature, the frequency of mimics is extremely low: fewer than 0.5% of anti-CD4 antibodies are structural mimics of gp120 and therefore the frequency with which anti-idiotypic antibody mimics of CD4 could be expected is less than $(0.005)^2$. The production of functional mimics bearing no structural similarity to the immunizing ligand can only be chance events of incalculable frequency. The B-cell repertoire is too large for us to argue that mimicry cannot occur but our data do indicate that the probability of success is discouragingly low. In accordance with this conclusion, for the 14 years since the anti-idiotypic approach was proposed, the criteria used by Erlan-

ger and others have been an insufficient basis for the isolation of novel receptors. As of October 1992, the SWISSPROT database lists at least 105 sequences for different human cellular receptors, none of which has been isolated with anti-idiotypic antibodies and shown to have the prescribed function. Clearly, for identifying and characterizing receptors, there are better methods than the anti-idiotypic approach.

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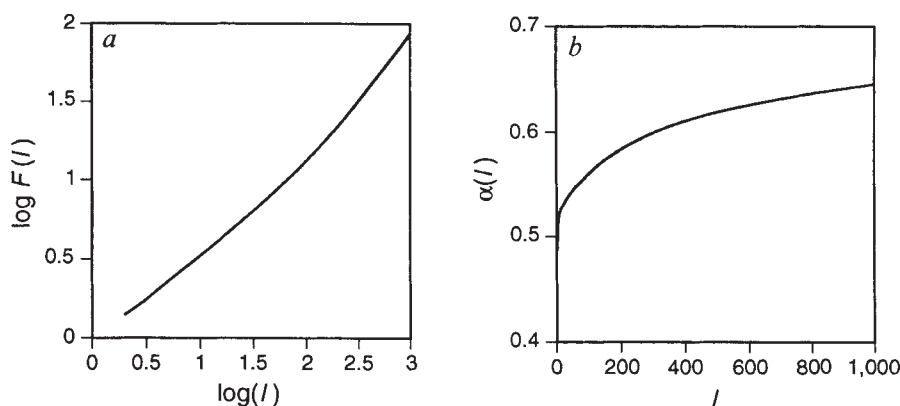
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Long-range correlations in DNA

SIR — Peng *et al.*¹ and Voss² provide convincing evidence for the existence of long-range correlations between bases in DNA sequences of living organisms, as originally shown by Li^{3,4}. The main results of these investigations seem to be inherently the same⁵, although the mathematical language used by Li and Voss may be less accessible to biologists than the method used by Peng *et al.* Therefore, we have used the method of Peng *et al.* to investigate these correlations. Our first analyses confirm the existence of certain long-range corre-

lations in DNA and reveal two new striking features.

Voss found differences in long-range correlations between sets of DNA sequences grouped as in the GenBank DNA database, ignoring the fact that the database categories are not of equal taxonomic rank. Peng *et al.*, in contrast, claimed that (regardless of taxonomic classification) intron-containing sequences (group A) display long-range correlations ($\alpha \sim 0.6$) whereas intronless sequences (group B) do not ($\alpha \sim 0.5$). Analysing the same data set as in ref. 1,



Plots of the function $F(l)$ (double logarithmic) for the bacteriophage λ (a) and the associated function $\alpha(l)$ (b) defined through $F(l) = l^{\alpha(l)}$. Adjacent data points are connected with straight lines as a guide to the eye. The value for α for each sequence is the arithmetic average of all $\alpha(l)$ values. $F(l)$ is a measure of the fluctuations of a 'DNA walk' defined as in ref. 1. With the abbreviation $\Delta y(l, l_0) = u(l_0+1) + \dots + u(l_0+l)$, with $u(i) = +1$ (-1) for a pyrimidine (purine) at the i -th position, one defines $F^2(l) = [\Delta y(l, l_0)]^2 - (l \langle \Delta y(l, l_0) \rangle^2)$, where the bars represent an average over all possible positions l_0 of a base in the sequence. $F(l)$ is the square root of $F^2(l)$. The significant distortion of the data in the log-log representation of $F(l)$ can be observed (see text). Thus the standard linear regression procedure gives a biased value of α if the considered graph is not a straight line. Therefore we calculated $\alpha(l)$ for $2 \leq l \leq l_{\max}$ and then built the arithmetic mean of all $\alpha(l)$ values, which defines the 'fractal power exponent' α . We chose $l_{\max}$ to be not larger than about one tenth of the DNA sequence under consideration¹. In most cases investigated, the function $\alpha(l)$ is not a constant, that is, there is not a well-defined scaling exponent.

we obtained values of α significantly larger than 0.5 for both categories. Indeed, only the human embryonic myosin heavy chain sequence lacks the long-range correlations ($\alpha = 0.47$). Thus, we find no consistent difference in α for the intron-containing versus the intronless sequences.

Owing to the considerable deviation of our data from those of ref. 1, we draw attention to the following. We tested the correctness of our computer program (which is based on the equations (1) and (2) of ref. 1) with artificial sequences of variable lengths, produced with the aid of a random-number generator, and obtained $\alpha \sim 0.5$ as expected. Second, we realized that the double-logarithmic (log-log) plot of the function $F(l)$ (as used in Fig. 2 of ref. 1) may easily distort some features of the displayed data. This is clearly illustrated by the two representations shown in the figure for the bacteriophage λ sequence (48,502 base pairs). Note that the log-log plot (a in the figure) gives the value $\alpha = 0.53$ (which coincides with the corresponding value of ref. 1) if one takes into account only the first 20–30 points of $F(l)$.

The figure also shows that the log-log plot of $F(l)$ exhibits a significant curvature, demonstrating that $\alpha(l)$ is not a constant. This interesting finding is characteristic for most of the DNA sequences investigated. Additionally, we find that most sequences exhibit a positive curvature (for example the bacteriophage λ sequence) whereas others exhibit a negative curvature. (Note that 15 of the 24 sequences analysed by Peng *et al.* encode myosin heavy chains, and that not even these share a similar value of α .) Thus we can conclude that a well-defined fractal power exponent α does not usually exist for a DNA sequence.

It has been proposed that the long-range correlations in intron-containing sequences may be caused by repeated segments in introns^{3–5}. Our results clearly demonstrate that long-range correlations are not unique to introns; see also refs 6 and 7. The explanation for these correlations in DNA sequences remains a challenge.

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Early T-cell development

SIR — The report by Molina *et al.*¹, showing a profound block in thymocyte development in mice lacking the p56^{lck} tyrosine kinase gene, deserves special attention. This work, as well as illustrating the crucial role of this T-cell specific tyrosine kinase in thymocyte differentiation, might also indicate the relevance of the expression of the β -chain of the interleukin-2 receptor gene during early T-cell development.

In T cells, p56^{lck} interacts and transduces signal(s) delivered through the β -chain of the receptor for interleukin-2 and through the CD4 and CD8 coreceptors². The impairment in thymocyte maturation in Lck-deficient mice reflects the inability of any of these molecules to transduce their signal(s). Because both CD4- and CD8-deficient mice have normal thymic architecture and no decrease in the number of thymocytes³, the most obvious candidate responsible for the observed phenotype in Lck-deficient mice is the interleukin-2 receptor β -chain. These three molecules (CD4, CD8 and the β -chain) are known to participate in T-cell differentiation. Although CD4 and CD8 signals are important during positive selection, the interleukin-2 receptor β -chain is thought to be involved in the prior phase of expansion of earlier thymocyte subpopulations. Thus, the interaction of interleukin-2 with the receptor β -chains constitutively expressed by freshly isolated prothymocytes promotes their differentiation into functionally competent T cells⁴.

We have previously reported the analysis of β -chain expression (in the absence of the α -chain) in the most immature T-cell precursors⁴. These studies revealed that pro-T cells isolated from neonatal thymus express higher amounts of β -chain molecules than mature T cells isolated from peripheral blood lymphocytes. By contrast, double-positive thymocytes (the stage at which positive selection of T cells takes place), have few, if any, β -chain molecules on the surface. Interestingly enough, only those differentiation stages characterized by the absence of expression of CD3, CD4 and CD8 and by the expression of the β -chain genes are the ones affected by the developmental blockage observed in Lck-deficient mice.

This, together with the fact that the developmental profile of expression of p56^{lck} parallels that of the interleukin-2 receptor β -chain gene during T-cell differentiation⁵, suggests that the phenotype seen in Lck-deficient mice is indicating the essential function(s) *in*

vivo of the β -chain molecules during the early expansion and differentiation phase of T-cell development. This view can only be confirmed, of course, by examination of β -chain deficient mice.

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Caliche and the carbon cycle

SIR — Kern and Schlesinger¹ infer that there would have been a decrease in carbonate carbon storage on land (as caliche/calcrete in soils) since the last glacial maximum, due to a substantial decrease in the global area of desert and semi-desert². They suggest that this may have offset part of the increase in organic carbon storage which seems to have occurred since that time. However, they do not point out that these two types of reservoir are not really comparable in terms of their roles in the global carbon cycle.

For example, there is a fundamental difference between the ways in which inorganic caliche and organic carbon tend to interact with atmospheric CO₂. An organic reservoir can release its carbon back into the atmosphere as CO₂ when it undergoes oxidation, while a caliche reservoir will tend to weather by taking up more CO₂ to form bicarbonate, when climatic conditions change to favour this. Thus, when a caliche reservoir shrinks due to weathering, it is mainly taking up CO₂ from the atmosphere (and contributing the resulting hydrogen carbonate, via river water, to the oceanic reservoir), while an organic carbon reservoir shrinking due to oxidation will tend to be releasing CO₂ into the atmosphere.

It is interesting to consider that at around the beginning of the present interglacial, both the shrinking caliche reservoir and the expanding organic reservoir would have been taking up CO₂ in parallel from the atmosphere. One can only guess at the quantitative and qualitative effects which these combined CO₂ sinks, together with the enhanced silicate and carbonate weathering sinks of glacial sediments and loess, had on the time course of the atmospheric CO₂

rise³ in the late glacial and early Holocene. However, from the bulk reservoir change of 1.35×10^{12} tonnes of carbon for terrestrial organic carbon inferred in ref. 2 and from the present-day global caliche carbonate estimate of 0.93×10^{12} tonnes inferred by Schlesinger⁴, it seems that CO₂ uptake may well have been of the same order as the rate at which CO₂ was leaving the oceans. It also seems advisable that those who wish to explain the time course of the atmospheric CO₂ rise during deglaciation seriously consider the role of the terrestrial system as a damping agent on any oceanically induced changes.

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Whither Pentastomida?

SIR — In his otherwise fine summary in News and Views¹ of the controversy surrounding limb homology and arthropod origins, Shear may have left readers with the impression that the enigmatic phylum Pentastomida still lies in limbo, awaiting a databased call to join the soon-to-be-erected umbrella phylum Lobopodia. Given their truly peculiar morphology, uncertainty over pentastomid affinities is understandable. However, Abele *et al.*² have recently provided molecular evidence that, among the taxa they examined (a crustacean, a chelicerate, a myriapod, an insect and an annelid worm), the pentastomids are most closely related to the subphylum Crustacea. Hence, rather than being an early proto-arthropod line of a rank comparable to onychophorans or tardigrades, as Shear implies, pentastomids fall, on molecular evidence, securely within the existing phylum Arthropoda, as some have argued all along^{3,4}.

Shear is to be commended for striving to keep a broad audience aware that the world of living invertebrates is still populated by weird and wonderful creatures whose affinities to other phyla remain obscure. One can only wonder in this age of increased concerns about biodiversity how many people have even heard of the Pentastomida, much less

have even a vague impression of what these animals look like. I'd be willing to bet a litre of fine Canadian rum that fewer than 1% of the readers of *Molecular Biology and Evolution*, where the original molecular data were published, and perhaps even of *Nature* itself, could say without running to an invertebrate zoology textbook whether members of this phylum swim or fly, jump or crawl,

Lipid–cytoskeleton interactions

SIR — Fukami *et al.* have shown the requirement of the acidic phospholipid phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) for α -actinin function¹. Among other lines of evidence for this notion, the authors find that endogenous and also added PtdInsP₂ remains bound to α -actinin even after SDS–PAGE and transfer to nitrocellulose. They conclude from this and by other methods that there is a very tight complex of PtdInsP₂ to α -actinin.

I have been working for some time on the interaction of lipids with the cytoskeletal protein vinculin, and it has been clearly established that this protein interacts with acidic phospholipids such as phosphatidylserine or phosphatidylinositol, as shown by hydrophobic photolabelling and gel-filtration chromatography^{2–4}. However, we could not find significant binding of ¹⁴C-labelled phosphatidylserine to vinculin after SDS–PAGE of a vinculin–lipid mixture. This is to be expected, as a strong detergent such as SDS is expected to dissociate non-covalent lipid–protein complexes.

Surprisingly, we found that substantial amounts of ¹⁴C-labelled phosphatidylinositol remained bound to vinculin after separation of a phosphatidylinositol–vinculin mixture by SDS–PAGE. This binding could, however, be prevented by inclusion of antioxidants such as mercaptoethanol or butylated hydroxytoluene in the medium during lipid sonication and subsequent incubation with vinculin, and by keeping the samples carefully under N₂. Moreover, 'binding' after SDS–PAGE was found only with certain batches of phosphatidylinositol (my unpublished observations).

These results point to covalent cross-linking of reactive lipid breakdown products to protein, due to oxidative deterioration of polyunsaturated fatty acid side chains. Specific crosslinking may occur as a result of a close protein–lipid interaction, and may depend on the degree of unsaturation of the fatty acids. Free radical trapping agents such as butylated hydroxytoluene prevent this crosslinking. Generally, care should therefore be taken to exclude oxidative processes when examining protein–lipid

chirp or sing, are brightly coloured or dull, or have legs, eyes, jaws or a tail, let alone have any thoughts on whether these animals should be placed within the Arthropoda or not. Any takers?

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interactions, and techniques other than SDS–PAGE should also be used, to substantiate the conclusions.

Fukami *et al.* present other lines of evidence for their conclusions, and I do not challenge them. But our experience shows that care is needed in evaluating lipid–protein interactions of this type.

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FUKAMI REPLIES — As Niggli mentions, phosphatidylinositol can bind to vinculin through its oxidized polyunsaturated fatty acid. Such binding is probably diminished in the presence of antioxidants such as mercaptoethanol or butylated hydroxytoluene. In our experiments¹, we carefully checked the binding of phosphatidylinositol 4,5-bisphosphate (PtdInsP₂) to α -actinin. The binding of PtdInsP₂ to α -actinin could be observed in the presence of 50 mM mercaptoethanol. Moreover, the binding was very specific to PtdInsP₂. We could not detect the binding of phosphatidylinositol or phosphatidylinositol 4-phosphate to α -actinin in western blot analysis. We also demonstrated the presence of PtdInsP₂-bound α -actinin even after striated muscles were solubilized directly by SDS sample buffer containing fresh mercaptoethanol. This binding seemed to be specific to α -actinin because we did not detect other PtdInsP₂-bound proteins by western blot analysis. Finally, we showed that PtdInsP₂ is specially localized in the Z-bands of striated muscle, where α -actinin is present. All these data suggest that PtdInsP₂ binds to α -actinin physiologically, though SDS–PAGE and western blot analysis alone may have some controversial aspects for the detection of lipids bound to proteins.

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Small is beautiful

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Taming the Atom: The Emergence of the Visible Microworld. By Hans Christian von Baeyer. Random House: 1992. Pp. 223. \$23. (To be published by Viking in the United Kingdom in March.)

TWENTY-FOUR centuries ago, the Greek philosopher Democritus and his teacher Leucippus gave birth to a new way of thinking about matter's invisible and elementary entity, the atom. Dem-

even speculated about the nature of atomic versus other forces. Important breakthroughs in physics early this century, together with earlier work that developed the concept of the chemical atom (by Dalton, for example) proved irrefutably the existence and structure of the atom. Von Baeyer points out the 'accidents' in the scientific approach, the acceptance with time of new scientific ideas, and the talent and intuition of some geniuses to see through complexity and devise incisive experiments or theory.

The atom was found to be divisible in 1897, when J. J. Thomson showed that the electron is a particle with a mass about two thousand times smaller than that of a hydrogen atom, the supposedly indivisible atom. With the discovery of the nucleus by Rutherford in 1910, the basic constituents of the atom were now known.

Work on the nature of light and its interactions with matter was crucial to this development, and some giant steps helped decipher the 'hieroglyphics of the atom' through the work of Maxwell (1873), Planck (1901), Einstein (1905), Bohr (1913) and de Broglie (1924). Soon after de Broglie's work, the wave nature of the electron was confirmed, by Davisson and Germer in 1925, by accident in a laboratory explosion, and by G. P. Thomson, the son of J. J. Thomson. At the same time, Heisenberg showed the importance of observables, matrix mechanics and the uncertainty principle. The following year, Schrödinger mathematically formulated wave mechanics and introduced the wavefunction concept, which Born, Heisenberg's professor, soon interpreted

in terms of probability (Dirac's work is not covered in the book). Newton's language for macroscopic systems was now replaced with a quantum language for microscopic systems. The new language challenged intuitive simple thinking — "Nobody understands quantum mechanics", Feynman once said.

But discoveries at atomic resolution in more recent times have brought the microscopic world and its language into a new age. In the bulk of the book, von Baeyer considers these discoveries (Chapters 4–9). He covers domains of spatial resolution and time resolution down to the scale of atomic distance (nanometre) and atomic time (femto-second) and describes the trapping and counting of a single atom and its interaction with the vacuum.

Spatial resolution at the atomic distance scale is illustrated by the scanning tunnelling microscope (STM), developed by G. Binnig and H. Rohrer in the 1980s. STM and its sibling AFM, the atomic force microscope, provide images of surfaces and of molecules on surfaces by tunnelling electrons through the materials or by 'feeling' them. The success of the method illustrates the beauty of a simple idea, in this case the osculation between a sharp needle and a flat surface with an atomic resolution. Although every chemist knows about the benzene molecule (discovered by Faraday in the 1820s) and about its ring structure (hypothesized by Kekule in the 1850s), only in 1988 was a direct visual image obtained of the molecule on a surface. With imaging techniques it is possible to 'view' the atomic landscape on the 'sensible' human scale. Colouring the atomic map becomes an architectural art.

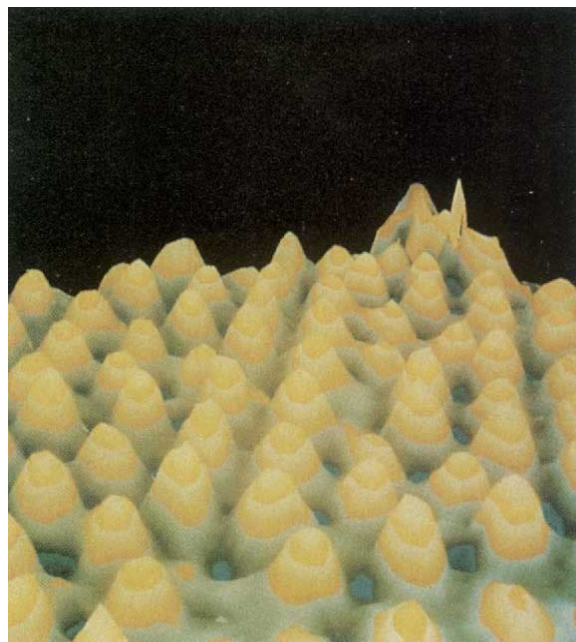


From *Taming the Atom*; courtesy D. Wineland

Atom trap — a charged mercury atom shows up as a little white dot in the centre of the video monitor. The other shapes are reflections off the trap components.

ocritus's atomism was born on a purely philosophical basis, surely without anticipating some of this century's most triumphant scientific discoveries — atoms can now be seen, seen in motion and manipulated. Von Baeyer vividly and eloquently describes the past, present and future of atomism. His account of present-day discoveries is largely centred on the three fundamental dimensions, space, time and number, and is written in a coherent and personal style. As Roald Hoffman notes on the dust jacket, the author has provided a striking verbal picture of the contemporary scene of physics and chemistry.

In the first three chapters, von Baeyer concentrates on the history of the atom, from ancient notions of its existence to our current understanding of its structure and language, quantum mechanics. The original ideas of Democritus and Leucippus were pursued in an attempt to unfold the complexity of simple phenomena in terms of a unifying and indivisible atom (from the Greek *a-tomos*, or 'uncuttable'). Aristotle rejected this atomism, but Newton accepted it and



From *Taming the Atom*; courtesy AT&T Archives

Surface impressions — silicon, magnified a billion fold.

Temporal resolution at the atomic time scale is illustrated by the femto-second stroboscope. Just as X-ray crystallography and electron diffraction, and now STM and NMR, provide distance resolution, so ultrafast lasers provide time resolution. Von Baeyer emphasizes the concepts in the development in 1985 of femtochemistry, drawing analogy between high-speed photography and the recording of the motions of atoms during a chemical reaction (reaction dynamics). The contrast with femtophysics is notable. Physicists identify the finest atomic distance, the scale of the nucleus, in units of fermis or femtometres equivalently. In femtochemistry, the unit is the femtosecond. For atoms in reactions, the femtosecond shutter speed is such that one can record the most elementary 'frozen' structure of the nuclei during the motion. On such ultrashort timescales newtonian mechanics may become the suitable language again. From Newton's earlier description of the motion to this century's quantum descriptions, von Baeyer illustrates the common and the elementary nature of dynamics and their influence on thermodynamics, electrodynamics, chromodynamics and reaction dynamics.

The single electron trap devised by H. Dehmelt in 1973 is a prime example of how single-particle resolution is achieved, and through the work of Dehmelt, D. Wineland, P. Toschek and others, von Baeyer relates the paradox of quantum jumps between levels of a single trapped and excited atom and the quantum theory of many atoms. In these traps, a single electron or atom is 'tamed' for days or even months, 'circling around' in a vacuum.

Democritus's old problem of atoms and the void can now be rephrased: what is vacuum made of and are the atom and the vacuum connected? The discovery in 1947 of the Lamb shift showed that the quantized energy levels of the atom reflect its interaction with the states of the vacuum. The Casimir effect (1948) was also important, but neither finding manipulated the vacuum. Fluctuations in the vacuum state are responsible for the spontaneous emission from an excited atom, and this phenomenon was exploited in D. Kleppner's idea of enhancing the spontaneous emission by changing the 'size of the vacuum' (1985). Concluding his discussions of present-day discoveries, von Baeyer devotes a chapter to the counting of atoms. The detection by S. Hurst in 1975 of single atoms using (multiphoton) ionization with a laser and counting the resulting electrons, makes it possible to reach new levels of sensitivity for applications in chemistry, geology and medicine.

What of the future? In the last five chapters, von Baeyer reflects on atomic

clocks and new standards for length and time, and on large-scale quantum mechanics and macroscopic phenomena such as superconductivity, superfluidity and atomic (or molecular) diffraction (the analogue of Young's diffraction with light, or electrons). Quantum-classical connections will continue to be revealed. Wave packets can already be created in atoms and molecules on an ultrashort timescale. Schrödinger would have been joyful about this, as in a letter to Lorentz in 1926, he emphasized the need for the construction of wave packets in order to connect quantum mechanics with newtonian mechanics. And questions remain. Can the laws of the 'very small' and 'very big' be unified and understood using the same language? Newton was troubled by the physical meaning of gravity, by interaction at a distance: can gravity waves be detected? What about chaos in the quantum world? What is the limit for subatomic particles? Can the manipulations of

Pencils of nature



"I HAVE captured a shadow!" exclaimed Henry Fox Talbot, the English mathematician and Assyriologist who invented a photographic process years before Louis Daguerre announced his own discovery in Paris in 1839. Talbot had developed a "photogenic drawing" method using paper treated with silver chloride (shown here is his salt print *Building silhouetted through trees*). But it was the eminent astronomer Sir John Herschel who devised the fixing process and gave the art its very name — photography. Herschel was also a highly skilled draughtsman, a skill

lacking in Talbot. Indeed, this deficiency motivated Talbot to invent photography — as a botanist and avid horticulturist, he was fascinated by the prospect of being able to draw plants "in a superhuman way". In *Out of the Shadows: Herschel, Talbot, and the Invention of Photography*, Larry J. Schaaf provides a detailed account of the relationship between the two men, the 'pre-history' of photography and the first five years of its public existence. The book is beautifully illustrated with more than 100 colour photographs. Yale University Press, £45, \$65. P.T.

atoms be extended to complex chemical systems? Can complex systems such as life molecules be understood on atomic and molecular levels?

Discoveries on these and other fronts will continue to be made; for the quantum atom, the discoveries will no longer prove its existence, structure or motion, but, as noted by John Wheeler, they will uncover the simple idea that demands the quantum. "I had rather discover one true explanation than be the King of Persia", Democritus said. If alive today, he would have enjoyed seeing the success of his philosophical idea of atomism and the discoveries made in physics and chemistry. Von Baeyer has provided an enjoyable and highly recommended account of these historic achievements. □

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Tools of our making

R. I. M. Dunbar

Chimpanzee Material Culture: Implications for Human Evolution. By W. C. McGrew. Cambridge University Press: 1992. Pp. 277. £40, \$79.95 (hbk); £16.95, \$27.95 (pbk).

CHIMPANZEES have long held a particular fascination for humans. Not only do they look more like us than does any other species of living animal, but research continues to uncover more and more behavioural and genetic similarities. McGrew's book focuses on just one feature of chimpanzee behaviour, namely those aspects of tool use that relate most closely to the only kinds of evidence that we have for the behaviour of our own direct ancestors.

The old definitions of 'Man the Tool-User' and 'Man the Tool-Maker' that served to distinguish us from brute nature were, of course, demolished two decades ago by evidence from wild populations: many animals (including sea otters and Egyptian geese) were found to use tools, and Jane Goodall's detailed studies of the chimpanzees at Gombe in Tanzania revealed that they also manufacture them. Further evidence of tool manufacture emerged from other long-term studies of chimpanzees in East and West Africa. Man's technological skills, it seems, were not unique.

Field biologists have always insisted that here was evidence for what in humans would be called culture. The suggestion has been greeted with less than enthusiasm by social scientists (and in particular anthropologists) who have tried to defend the uniqueness of humans, if necessary by moving the goal posts.

McGrew has sought to confront the issue directly by using the criteria that social anthropologists themselves use to identify culture. Chimpanzees make use of the same medicinal herbs that indigenous peoples do; they hunt in co-ordinated forays; and they show regional variations in diet that bear no relationship to the local ecology but that seem, instead, to be local traditions.

He uses Oswalt's taxonomy of tools to compare the chimpanzee's toolkit with those of the native Tasmanians (who until their extinction had the most primitive toolkit of all living humans) and other living hunter-gatherers, as well as with the 'fossil' toolkits left behind by our extinct early ancestors. They are not so different in the number or the complexity of the tools. "Some artefacts", he

observes, "would be unattributable to species [that is, human or chimpanzee] if they lost their museum labels."

One important point to emerge from this analysis is that many of the tools in the toolkit of living hunter-gatherers are made from vegetable matter: such tools will not be fossilized. Our hominid ancestors are therefore likely to have made and used tools for millennia before the appearance of the first stone tools at around 2.5 million years ago. Archaeologists have had an all but total fixation with stone tools, using their appearance as the benchmark against which to measure the cognitive progression of the hominid lineage.

Only with respect to two items do the toolkits of chimpanzees and humans differ. One is the use that humans make of containers and nets for transporting and storing food and of 'untended facilities' such as traps and snares. For the rest, there are, of course, differences but they are differences of quantity not kind.

The gap between chimpanzees and humans is decidedly bridgeable. It would not take an enormous intellectual step by some chimpanzee Einstein, for example, to extend this species' natural preparation of foods to cooking.

This book is an important contribution both to our understanding of chimpanzee behaviour (it provides the first detailed critical synthesis of a widely scattered literature of very variable quality) and to our understanding of the processes of hominization. It will be particularly valuable if it persuades social scientists to take the work of primatologists and other animal biologists more seriously. □

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■ Oxford University Press have just published *Biology, Rearing, and Care of Young Primates* by J. K. Kirkwood and K. Stathatos. £40, \$75.

Undescribed experiences

Olivia Cox-Fill

The Change: Women, Ageing and the Menopause. By Germaine Greer. Hamish Hamilton/Knopf: 1991/1992. Pp. 472. £15.99, \$24. (Also published in paperback by Penguin in the United Kingdom, £6.99.)

THIS masterly book examines the social, physical and emotional factors surrounding the menopause. In a voice that is at once humane, angry, witty and totally uncompromising, Greer tirelessly explains the upheavals that come with the cessation of ovarian function. It is an intensely personal work, yet scholarly in its attempt to diagnose the climacteric and the best treatment of its painful symptoms.

Greer reminds us that the climacteric syndrome is ill-defined: 'experts' don't know exactly what happens or why or when it happens, and they cannot even yet convincingly disentangle the symptoms from ageing. She is thus frustrated by the smoke-screen of ignorance raised by those with interests to protect, and angry at the resulting trivialization of women's lives — the sense of helplessness and powerlessness that they are forced to buy into at the hands of those she calls the Masters of Menopause. Indeed, a multi-million-dollar industry has been established to treat what is in effect "an undescribed experience".

Officially, the medical establishment has one treatment for the climacteric, and that is hormone replacement therapy (HRT). Endocrines have been used

to treat menopausal distress ever since natural oestrogens were first isolated in the 1920s. Although these substances had dubious biochemical action, they did develop some entrepreneurial activity. Greer traces the history of the menopause industry and mercilessly lambasts the multinational pharmaceutical companies for their sham in relentlessly developing and marketing "hormone cocktails". She questions the premise of their research and is highly critical of the results. Nonetheless, her discussion of the costs, benefits and risks of HRT is balanced and fair, and her examination of the possible harmful physical effects of progestones, which are usually administered to oppose the effect of the oestrogens on the lining of the womb, is important reading. The crux of the matter is that research into the menopause has been lackadaisical because 'the change' is not a life-threatening condition.

The medical profession has also contributed its share of ignorance, through its female as well as male practitioners, whose contradictory claims Greer exposes. She also describes her exhaustive search of scientific papers and of transcripts of meetings organized to establish the international market for HRT. The aim of many of them, she complains, was to secure government funding "to spread the gospel of HRT into every hovel on the planet", not on the basis of the therapy's benign effect on menopause, but because of its alleged beneficial effect on osteoporosis and the

ageing process of women's sexuality.

Some women seem to ignore the symptoms of the climacteric. Greer argues that this has more to do with their coping style than the extent to which they experience symptoms. Many older women find satisfaction where men do — in their relations with their employer, and in the feeling of well-being that comes from mastering their job. Greer muses wistfully on how much better women would feel during the menopause if, instead of losing prestige, power and responsibility, they were to gain all three, as they do in matrilineal societies of the so-called developing countries.

Greer defends the right of women in their 50s to their mood-swings. After the years of silent rage endured as the family's doormat, many women suddenly find that they are poor, dependent, insecure and lonely, and they should not have to feel guilty about their justified moodiness as well; by contrast, the husband usually has the social continuity of his career to sustain him and is supported by a society that finds older men more attractive than women of the same age. In particular, women should be allowed to grieve when "the day has passed its noon and the shadows are growing ever shorter and bleaker"; and she tells us that they must demand their own space so that they may make the final part of their lives as rewarding as possible. She encourages them to redefine their image and develop reasonable expectations about the climacteric.

The book contains a perceptive examination of historical and fictional stereotypes of ageing women. And as a final bonus, there is a charming chapter in which we are treated to the poetry and biographical details of some of the great women survivors. Karen Blixen was 46 when she returned from Africa to her native Denmark with spinal syphilis and a life in turmoil. To aid her recovery, she fashioned herself as a writer under the name of Isak Dinesen: *Out of Africa* was to be her masterpiece, but in an earlier book she describes a woman that could as well have been herself or even the author of *The Change*:

What changed her was what changes all women at fifty; the transfer from the active service of life — with a pension or the honours of war, as the case may be — to the mere passive state of a looker on. A weight fell away from her; she flew up to a higher perch and cackled a little.

Greer's hope that all women will one day gain prestige at the time of the climacteric is unrealizable. But on reading this informative book they will at least know that they are not alone. □

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Making bucks

Thomas W. Ebbesen

How to Profit from Fullerenes: Winning Strategies for Emerging Markets. *Technical Insights: 1992.* Pp. 165. \$1,225 (\$1,275 outside North America). (For further information, contact P. Finlay, Technical Insights, PO Box 1304, Fort Lee, New Jersey 07024-9967, USA.)

WHEN experimental evidence for the existence of fullerenes (C_{60} and C_{70}) was reported in 1985 (H. W. Kroto *et al.*, *Nature* **318**, 162; 1985), it sparked the imagination of scientists worldwide. If fullerenes really existed, they would form a unique new class of material — the first all-carbon molecules, spherical, hollow, aromatic and highly symmetric. So unique properties were expected, but there was no way to find out, because only trace amounts of the material were present in the mass spectrometer. Theoreticians got to work and predicted, among other things, the simple four-peak infrared absorption spectrum of C_{60} . The breakthrough came in 1991, when W. Kratschmer and D. R. Huffman noticed the same spectrum for the soot they had generated in carbon-arc experiments, from which they eventually extracted C_{60} (D. R. Huffman, *Physics Today*, November 22–29; 1991).

This simple experiment finally made fullerenes available to everyone, in milligram to gram quantities. It resulted in an explosion of research activity, from organic chemistry to solid-state physics. Within a year, the prediction that C_{60} was a unique new material was confirmed, most spectacularly perhaps by the discovery of its superconductivity at AT&T Bell Laboratories.

So in this context of excitement and broad interest, it is not surprising that there is a market for a book on the potential of fullerenes in the marketplace. Indeed, a lot of companies have invested in fullerene research and others are considering following suit. *How to Profit from Fullerenes* is stimulating and should provide a useful overview for business people. It points out, probably correctly, where the market potential of fullerenes is to be found; but sadly it is spoiled by many scientific, and sometimes historical, inaccuracies.

The book starts with an "executive summary" of the contents, which indeed should be useful enough for busy administrators. But it is not always consistent with the later chapters. This is of course a problem in a rapidly evolving field where breakthroughs are made monthly. For instance, carbon nanotubes (alias buckytubes) are described first in terms of whether or not they exist and then in

terms of their availability in usable quantities. At the end of the book their large-scale synthesis is reported.

Busy readers should also take the time to look through the first two chapters, which give a good short perspective on fullerenes. I would agree with the statement that collaborative research is probably the most effective path to take because, with the rapid pace of development in fullerene research, "no single group can take full advantage of every salient discovery". That has been clearly observed. It also seems true that fullerenes are not just a fad but a durable and expanding field, especially with the prospects for 'customized', chemically modified fullerenes.

Chapter 3, "Properties, Synthesis and Purification", gives an introduction to the chemical kinetics and has far too much detail for nonscientists, but is insufficient and too inaccurate for scientists. Chapter 4 discusses chemically modified fullerenes in the broadest sense and is useful for nonspecialists. Here it is again argued convincingly that chemical modification of fullerenes into a high-tech material has the greatest potential, giving them enough added value to cover the high cost of raw fullerene material. (At about a billion dollars a ton, it is certainly unlikely that C_{60} will compete in, say, the rocket fuel market, as some have suggested.)

In Chapter 5, potential applications markets in which fullerenes would compete are discussed. Areas such as superconductivity, nonlinear optics, diamond making and chromatography are covered. Perhaps the most intriguing story is the invention of rhondite, an iron-fullerene blend analogue of steel which under the proper heat treatment turns fullerene carbon into diamond, resulting in so-called 'diasteel'. The least explored territory is the use of fullerene-based materials in pharmaceuticals and medicine (for example in immunoassays). The report ends with a very partial listing of researchers worldwide involved in fullerene development projects.

The US Patent Office apparently now receives more correspondence on fullerenes than on all other inventions combined. Although their true potential remains unknown, there is no question that fullerene-based materials will provide opportunities not to be missed. □

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■ Newly published is *Macmillan's Chemical and Physical Data* by A. M. James and M. P. Lord, a rich and concise source of fundamental data in 17 well-defined subject areas, from atomic and nuclear physics to acoustics. Macmillan, £35.

The fossil record and the early evolution of the Metazoa

S. Conway Morris

The appearance of the multicellular animals, or Metazoa, in the fossil record about 600 million years ago marks a revolution in the history of life. Molecular biology is continuing to increase our understanding of metazoan evolution, yet information from fossils is still an important component in deciphering metazoan phylogeny, and data on rapidly radiating animal groups place early metazoan evolution in a new perspective.

WOULD you like a game of 'Metazoan Phylogeny'? The prospect is daunting. The board encompasses the entire Earth: the only game ever attempted has already taken almost a billion years and shows no signs of a conclusion. The number of players varies (around 35 at the last count), and the strategic implications of such rules as may exist have not been fully worked out. Ideas about metazoan phylogeny are legion and often contradictory¹⁻⁴. A few principles are widely, but not universally, accepted, but no coherent phylogeny for the roughly 35 metazoan phyla exists. Speculation has been based mostly on anatomy, both adult and larval; meanwhile ultrastructural studies, immunology and biochemistry have added to the debate, and sometimes the confusion. Now, the picture has changed for ever. The true outlines of metazoan phylogeny seem to be emerging. This is due to advances in molecular biology, most notably data from ribosomal RNA⁵⁻⁸.

If the broad outline of metazoan phylogeny is now becoming clear, then surely all we need to do is commiserate with the authors of innumerable failed schemes constructed over the past century and promptly move on to more interesting problems. This would be a mistake, for three reasons.

First, the power of molecular phylogeny is not unlimited. If diversification is rapid, then the precise order of branching is very difficult to resolve^{8,9}. At present, unresolved branchings (polychotomies) persist, especially in triploblastic protostomes⁶.

Second, the new schemes of metazoan phylogeny tell us nothing about the actual anatomical and functional transitions between related phyla. For instance, molecular evidence¹⁰ suggests a close alliance between molluscs and annelids, but it is hard to imagine how an animal like the most primitive known annelid (an archiannelid?) was transformed into a primitive mollusc (a chiton?). The morphological gaps that, by definition, separate phyla, remain inviolate. We remain uninformed both about the now-extinct intermediates and the evolutionary processes that would have been responsible for the diversification of early multicellular animals into what we now perceive as distinct phyla, each with its own body plan.

Third, the new view of metazoan phylogeny requires a radical review of existing data, especially about ultrastructure, functional anatomy and biomineralization. Convergent evolution is likely to have been rampant.

If these problems can be solved, we would have an enhanced understanding of the constraints that guide evolutionary processes, the role of intrinsic and extrinsic factors in diversification, and a new appreciation of metazoan diversity: in short, the rules of the game.

My purpose here is to review how the fossil record of early metazoans might be integrated with the new phylogeny. Three types of fossil deposit yield relevant information: Ediacaran-type assemblages of soft-bodied animals from the latest Precambrian (Vendian), typified by the Ediacara fauna of South Australia; the earliest assemblages of animals with hard parts, from the Lower Cambrian; and the distinctive faunas from the Lower

and Middle Cambrian, exemplified by the Burgess Shale of western Canada (Fig. 1). All these deposits have attracted wide attention because of the apparent abundance of novel body plans. These so-called 'bizarre' fossils may help to unravel the basic structure of metazoan evolution (Fig. 2).

Pre-Ediacaran metazoans?

When did metazoans appear? The objective fossil record starts with the first Ediacaran assemblages (~560–600 million years (Myr) ago), but some evidence suggests that metazoans were already in existence as early as ~800–1000 Myr ago. Several lines of evidence indicate that metazoans originated at around that time, such as a decline in the diversity of stromatolites, possibly indicative of grazing and burrowing of the microbial-mat communities by metazoans¹¹. Other explanations for stromatolite decline, however, are also plausible. These include changes in ocean chemistry¹² or the microbial mats adapting to the evolution and arrival of a wide range of new types of protistan¹³. Second, trace fossils, including what may be metazoan faecal pellets¹⁴, are found in rocks of this age. But protistans can produce structures similar to metazoan faecal pellets¹⁵, and no systematic survey for pre-Ediacaran traces, including bioturbation fabrics^{16,17} and geochemical anomalies around possible burrows^{18,19}, has been undertaken. Third, evidence from molecular biology^{20,21} indicates that the major lineages of Metazoa were distinct at least 700 Myr ago (suggestive of even earlier origins), but this too is disputed²².

Molecular evidence indicates a major diversification of eukaryotes⁸ perhaps ~1,000 Myr ago^{13,23}. Were metazoans part of that evolutionary event? Millimetres in size, perhaps interstitial in sediments, their inability to fossilize or leave obvious traces could explain their absence from Proterozoic rocks. The reconciliation of this idea with what is known of subsequent evolutionary events remains problematic (Fig. 1). If triploblasts (metazoans with three germ layers) occur in Ediacaran assemblages, then perhaps they too had an extended history in parallel with early diploblasts (two-layered animals such as modern coelenterates). Why the first, albeit hypothetical, metazoans never grew beyond a few millimetres in size is not understood. A persistently low concentration of atmospheric oxygen is one possible explanation, but alternatives include the late acquisition of collagen (not unconnected with the oxygen-concentration idea²⁴).

Ediacaran metazoans

The soft-bodied Ediacaran animals occupied a wide range of Vendian (Latest Proterozoic) marine environments and are now known from all over the world. Many of the fossils are generally interpreted as coelenterates, and some segmented forms as arthropods, annelids or other triploblasts. Nevertheless, Ediacaran fossils may not be representative of all Vendian metazoans. A newly described assemblage²⁵ from black shales in the Doushantuo Formation (Yangtze Gorges) in China

includes tubular fossils that might have been occupied by metazoans. The presence of fossils of seaweeds from the same deposits suggests that the remains of soft-bodied animals, not just their traces, will be found there in the future.

Molecular evidence is also consistent with the early appearance of coelenterates (cnidarians and ctenophores)⁶, but probably not independently of other metazoans²⁶. Only sponges⁸ and the microscopic placozoans may have branched off earlier. The chance of placozoans fossilizing is minuscule, but the apparent absence of Precambrian sponges is puzzling. Siliceous spicules of demosponges occur in strata in Iran²⁷ that may be of Ediacaran age, and claims for Precambrian sponge spicules²⁸ need re-examination, especially as protistan biomineralization²⁹, possibly of silica, is known from the preceding Riphean Period (Fig. 1)³⁰.

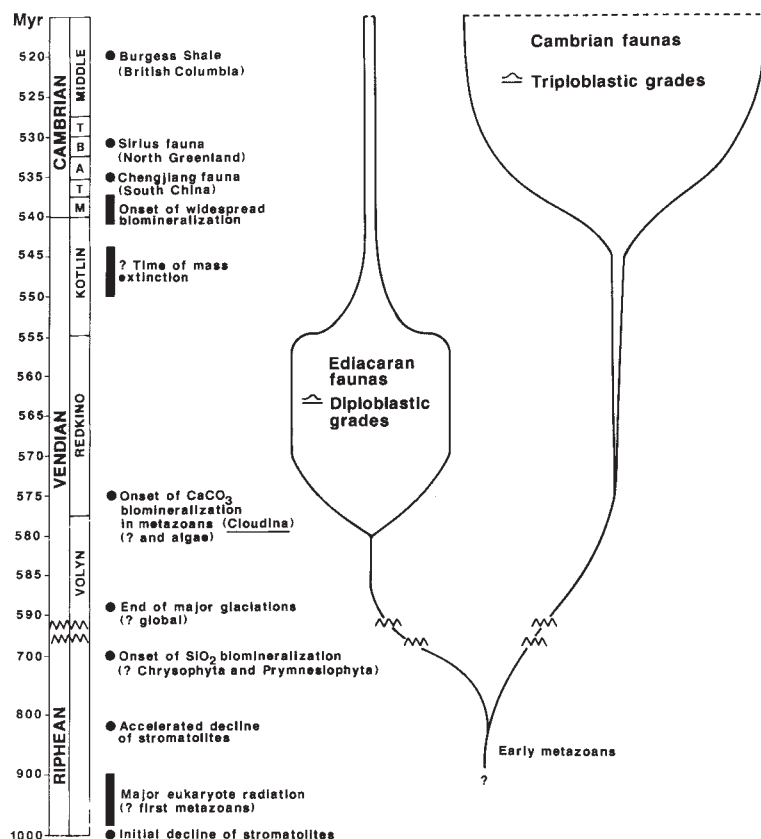
The primitive position of coelenterates⁴⁻⁸ accords well with the domination of the Vendian radiation by diploblastic animals. This supposition³¹ has been overshadowed by the 'Vendobionta' hypothesis^{32,33}, in which Ediacaran animals are thought to constitute an extinct clade of multicellular eukaryotes, neither diploblast nor triploblast, with a unique construction of tough cuticle and mattress-like body, and possibly the absence of alimentary tract, musculature and nervous tissue. This concept is open to debate, in that at least some Ediacaran fossils can be compared with known metazoans^{31,34}. *Inaria*, for example, may be an actinian coral³⁵, and the fivefold arrangement of putative feeding grooves in the minute epibenthic *Arkurua* suggests an affinity with echinoderms³⁶. Other organisms show evidence for muscular activity³⁴ and so presumably a nervous system, as well as the inferred presence of a circulatory system. An analogue of Ediacaran organization may be found in the modern deep-water scleractinian coral *Leptoseris fragilis*: instead of tentacles, it uses its ciliated surface as a food-gathering organ, and has an internal canal-like gastrovascular system that communicates with the exterior through pores³⁷. So is the Vendobionta concept completely redundant? Not necessarily, because the Proterozoic may

have witnessed the achievement of the multicellular grade of organization several times, and in quite unrelated lineages.

As for coelenterates, though, several arguments suggest that all four extant cnidarian classes can be recognized in early metazoan assemblages. Anthozoans are represented by pennatulacean-like fossils³⁸ such as the Ediacaran *Charniodiscus*. Relatives in the Burgess Shale show what may be zooids³⁹. Actinians may have occupied bowl-like fossils such as the Vendian *Belanelliformis*^{40,41} and the Cambrian *Berguaria*⁴². Biomineralization of cnidarians produced a variety of primitive coral-like fossils⁴³. *Kimberella* may be a cubozoan³⁸, represented today by the venomous box-jellies, whereas forms such as *Ovatostrotum*³⁸ may represent the floats of hydrozoans, as in the present-day Portuguese man-of-war. Jellyfish-like Ediacaran forms have been compared with 'true' jellyfish, or scyphozoans, but there the evidence is more tenuous.

Probably related to the scyphozoans are the enigmatic conulariids, with ribbed phosphatic tests and a characteristic tetra-radial symmetry. The Lower Cambrian carinacitiids (Fig. 3f) and hexagulaconulariids⁴⁴ are probably conulariids, and the Ediacaran *Conomedusites*⁴⁵ may also belong in this group. The xianguangiids⁴⁶, from the Burgess Shale-type fauna at Chengjiang in China (Fig. 1), are interpreted as anthozoan-like cnidarians on account of a basal disc, polyp-like body with possible septal impressions, and a distal crown of tentacles. The tentacles, however, are unique in bearing closely spaced pinules, a feature overlooked in the original description⁴⁶. One group of Ediacaran medusiforms is characterized by tri-radial symmetry. Cambrian descendants of these so-called trilobochoans (such as *Albimares* and *Tribrachidium*; Fig. 3a) may be represented by the anabaritids^{47,48}; their calcareous tubes are tri-radial (Fig. 3d), as defined by internal keels (Fig. 3b, e). Rare branching in anabaritid tubes⁴⁹ is consistent with a cnidarian grade. Cnidarians may also be represented by the tube-like byroniids⁴⁸, the possibly tentacle-bearing *Cambrotrichium*⁵⁰, the paucitids with septate tubes⁵¹, and conceivably the tubular

FIG. 1 The geological framework of early metazoan evolution and related biological events. The geochronological scale takes the Precambrian-Cambrian boundary at ~540 Myr ago¹¹² and the Ediacaran faunas as ~580-560 Myr ago. The stratigraphic divisions of the Vendian and Lower Cambrian (M, Manykay; T, Tommotian; A, Atdabanian; B, Botomian; T, Toyonian) follow the Russian and Siberian standards. Metazoans are hypothesized to have arisen as part of a major eukaryotic radiation, ~800-1,000 Myr ago^{13,23}. Tangible fossil evidence includes the Ediacaran radiations of mostly cnidarian grade, but also stem protostomes and possible deuterostomes. The 'Cambrian explosion' is largely a triploblastic radiation, but diploblasts and triploblasts may have diverged substantially before Ediacaran times.



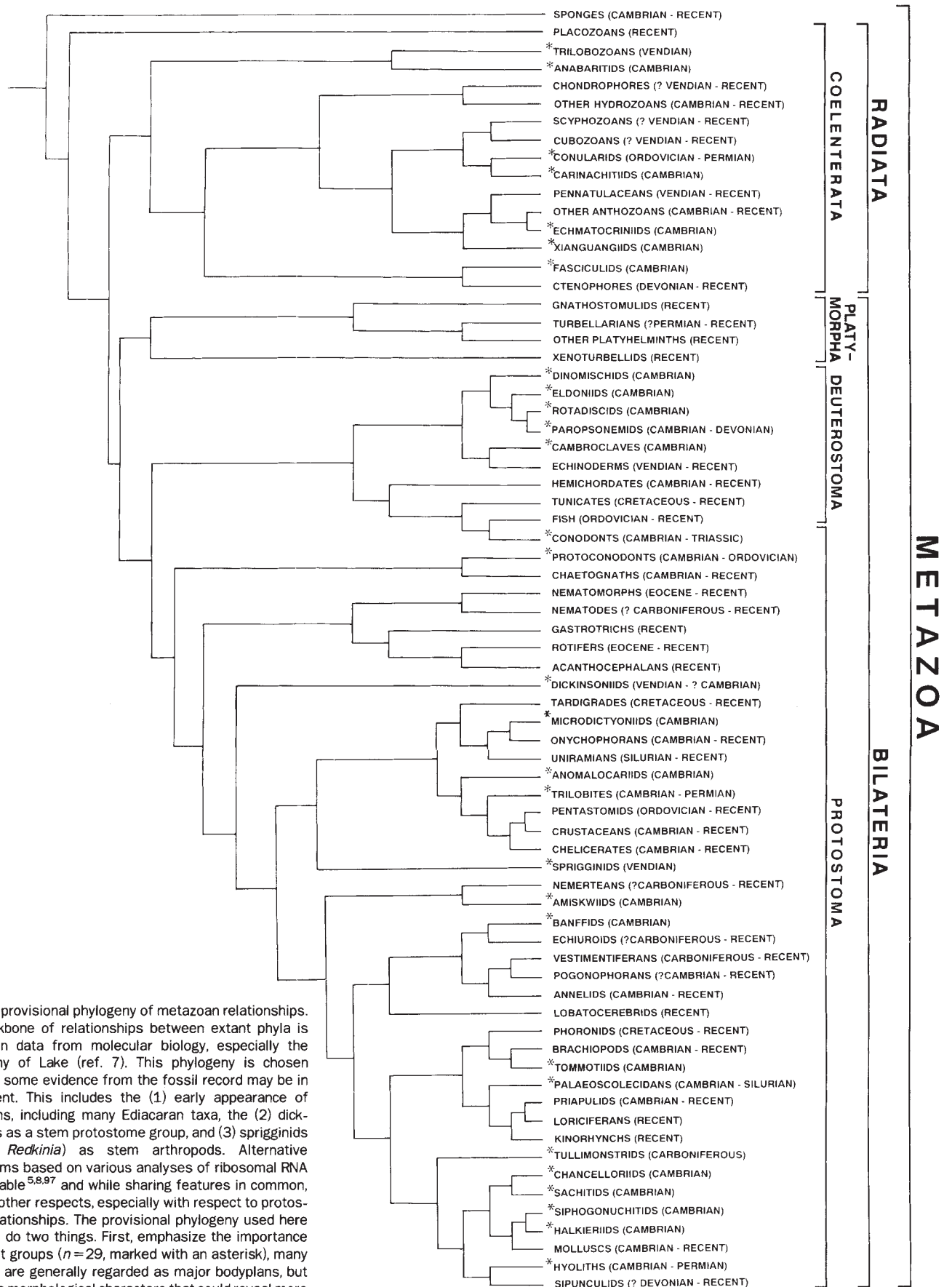


FIG. 2 A provisional phylogeny of metazoan relationships. The backbone of relationships between extant phyla is based on data from molecular biology, especially the phylogeny of Lake (ref. 7). This phylogeny is chosen because some evidence from the fossil record may be in agreement. This includes the (1) early appearance of cnidarians, including many Ediacaran taxa, the (2) dickinsoniids as a stem protostome group, and (3) sprigginids (? and *Redkinia*) as stem arthropods. Alternative cladograms based on various analyses of ribosomal RNA are available^{5,8,97} and while sharing features in common, differ in other respects, especially with respect to protostome relationships. The provisional phylogeny used here seeks to do two things. First, emphasize the importance of extinct groups ($n=29$, marked with an asterisk), many of which are generally regarded as major bodyplans, but may have morphological characters that could reveal more clearly relationships between disparate extant phyla. Second, this cladogram is an exercise in inference because many of the extinct groups remain poorly documented; the text offers justification for many of the placements. Note that the levels of branching are arbitrary and no precise metric is

applied to distance between the nodes. A number of clades are still too poorly known to be included. These include mobergelliids, rhombocorniculids, nectocariids, cribricyathids, radiocyathids, escumasiids, myoscolexids, coleoliids, agmatans, typhloesids and ainiktozooids.

cloudinids. The last constitute an important Vendian group⁵², and are among the first metazoans to have acquired hard parts. Another candidate is the Burgess Shale *Echmatocrinus*, currently interpreted not as a cnidarian at all, but as an echinoderm, the earliest crinoid⁵³: the supposed tube feet are very large and could instead be anthozoan-like tentacles. In contrast, *Echmatocrinus* had plated structures on its polyp and tentacles but, unlike the co-occurring eocrinoids⁵³, the plates appear not to show a stereom structure.

The sister group of the Cnidaria is the Ctenophora, the comb-jellies. A pelagic habit and a delicate, gelatinous body account for their poor fossil record. *Fasciculus*⁵⁴, from the Burgess Shales and adjacent localities, is an early ctenophore, but differs from extant and Devonian ctenophores⁵⁵ in the number of comb-rows.

Triploblasts inherit the Earth

If Ediacaran assemblages are dominated by coelenterates, the ensuing Cambrian radiations are largely the irruption of triploblastic phyla. There are essentially two kinds of coelomate triploblastic metazoans: the protostomes and the deuterostomes. Protostomes, such as annelids and arthropods, are usually regarded as having spiral, determinate embryonic cleavage, and the embryonic blastopore is situated at the rear, at or near the site of the future mouth. Deuterostomes, in contrast (principally echinoderms and chordates) generally have radial, indeterminate cleavage, and the blastopore is associated with the position of the adult anus⁴.

Fossil evidence for the 'Cambrian explosion'⁵⁶⁻⁵⁹ is evident from the widespread rise of animal skeletons^{48,49}, the major diversification of trace fossils⁶⁰ and the Burgess Shale-type faunas⁶¹. But it would be unwise to draw too strong a line between Ediacaran and Cambrian faunas. The Ediacaran *Dickinsonia* may be a stem-group protostome: it is segmented, bilaterally symmetrical and has a clear anteroposterior axis^{31,34}. Other Ediacaran taxa could also be protostomes. *Spriggina* (Fig. 4e) could be a stem arthropod, and *Redkinia*⁵⁹ could represent mandible-like jaws: and if *Arkurua*³⁶ is interpreted as an echinoderm, the deuterostome lineage is also present in Ediacaran times.

Triploblastic diversification was rapid on a geological time-scale^{6,8}, and resolution of the branching order is consequently difficult. Refined stratigraphy, including recent advances in chemo- and magnetostratigraphy⁶², may resolve some orders of appearance on a global scale. As far as fossils go, though, local taphonomic bias in favour of the preservation of either articulated scale-like armour⁶³, phosphatized skeletal fragments^{48,49} or soft parts⁶¹ leads to a sporadic and incomplete record.

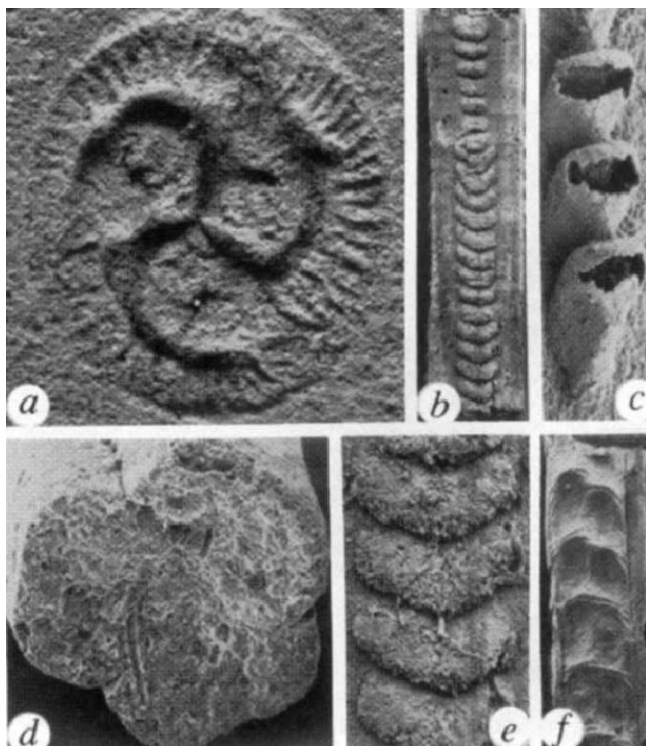
The 'Cambrian explosion' is a real evolutionary event, but its origins are obscure. At least 20 hypotheses have been proposed, and although arguments linking diversification to oxygen levels, predation, faunal provinciality and ocean chemistry all attract support, it is the case that 'The emergence of Metazoa remains the salient mystery in the history of life' (p. 17, ref. 58).

Deuterostome radiations

Molecular data⁶⁻⁸ suggest a profound evolutionary gulf between deuterostomes and protostomes. Among the Cambrian deuterostomes, the echinoderms have a good fossil record, and the inter-relationships between many of the supposedly disparate classes are becoming clearer⁶⁴. If the characteristically echinoderm skeleton of calcareous 'stereom' was acquired before the equally distinctive water-vascular system, then the aberrant cornutes (which have the first, but not the second) are a very early branch of echinoderms. The chordate-like features of cornutes, which have been proposed to indicate a close relationship with chordates⁶⁵, arose by convergence. The most primitive echinoderm with a water-vascular system appears to have been *Helicoplacus*, notable in having three rather than five ambulacra⁶⁴. These observations need to be reconciled with the pentaradial arrangement in the Ediacaran *Arkurua*³⁶, and also suggest an alternative assignment for trilobozoans (Fig. 3a). The cambroclaves may be allied to echinoderms, albeit more tentatively, because of their arm-like arrays^{66,67} of articulated sclerites (Fig. 3c). These structures seem to suggest a sessile, echinoderm-like form rather than the creeping, slug-like habit previously suggested for these animals⁶⁷.

Chordates appear with *Pikaia* from the Burgess Shale⁶¹. This creature superficially resembled the modern amphioxus and had

FIG. 3 Vendian and Cambrian metazoans. a, *Tribrachidium heraldicum*, a discoidal Ediacaran fossil with three 'arms' from the Pound Subgroup, South Australia; magnification, 2.9×. *Tribrachidium* has been attributed to the trilobozoans (a possible group of early cnidarians) or alternatively a protoechinoderm with the tri-radial symmetry preceding the penta-radial arrangement of most later echinoderms. b, e, *Anabarites compositus* from the Lower Cambrian of northern Siberia showing steinkern of tube (b; ×50) and detail of porous blade-like extensions (e, ×200) that arise in three rows along the interior wall of the tube. (Specimen kindly made available by V.V. Missarzhevskiy.) c, *Deltaclavus graneus*, a series of articulated sclerites from the Lower Cambrian of Hubei, China, ×90. More complex arrays of sclerites have an 'arm-like' structure that could indicate a relationship to echinoderms (see Fig. 2). d, *Anabarites trymatus* from the Lower Cambrian of south Australia, steinkern showing triradial symmetry of tube; magnification, 150×. f, *Carinachites spinatus*, an early ?cnulariid (Cnidaria) from the Lower Cambrian of Shaanxi, China; magnification, 36×.



a bilobed head and pair of tentacles, as well as myotomes and a notochord which, unlike the amphioxus, appears not to have extended to the anterior. Gill slits may have been present, but are hard to identify with certainty in the compressed material available. Apart from conodonts⁶⁸, unequivocal evidence for fish does not occur until the Ordovician⁶⁹. In particular, the fossil *Anatolepis*, which first occurs in the Upper Cambrian and has been interpreted as the dermal scales of a heterostracan fish⁷⁰, is more likely to have belonged to an arthropod⁷¹.

The record of hemichordates is moderately good^{72,73}; the organic tubes of rhabdopleurid pterobranchs have a fair preservation potential, and the Burgess Shale '*Ottoia*' *tenuis* may be an enteropneust similar to the extant acorn-worm *Balanoglossus* (unpublished observations by S.C.M.).

The nature of the deuterostome ancestor is speculative. The eldoniids⁷⁴, a group of medusiform animals each with a prominent coiled gut (Fig. 4a) may repay consideration in this regard. They could represent a group of pre-echinoderm deuterostomes, as neither stereom nor a true water-vascular system appears to be present. Groups that may be related to the eldoniids include rotadiscids⁷⁵, paropsonemids⁷⁶ and *Velumbrella*⁷⁷.

Protostome radiations

Platyhelminthes appear to be the most primitive triploblasts but, apart from possible trace fossils⁷⁸, their fossil record is almost non-existent. Again, fossils have little to say about the aschelminthes (nematodes, rotifers and others) which may, in any case, be a polyphyletic grade. The nematodes and the related nematomorphs may be relatively primitive, but the present proximity of groups such as the rotifers and the related acanthocephalans⁷⁹ could require revision.

The branching pattern of the remaining protostomes is just as contentious. Lake⁷ upset established orthodoxy by arguing that the arthropods are paraphyletic, arising before annelids and molluscs. In contrast, R.A. Raff (personal communication) regards arthropods as the sister group of molluscs–annelids–brachiopods–pogonophorans, the latter assemblage arising as an unresolved polychotomy.

Arthropods and pre-arthropods. The fossil record of Cambrian arthropods is moderately good, and even the riot of supposedly disparate forms from the Burgess Shales seem to fall into a phylogenetically coherent scheme. The available cladograms⁸⁰ are as yet tentative, but as information about arthropods from the Burgess Shale-like Chengjiang⁸¹ and the Sirius Passet fauna from northern Greenland becomes available, cladistic analyses should improve.

More important is the nature of the pre-arthropod stocks. Apart from *Spriggina* (and possibly *Redkinia*), the anomalocariids⁸² currently attract much interest. They are very diverse, but they all have prominent lobate appendages, a well developed head with eyes, and sometimes a tail-fan. Undescribed material from localities adjacent to the Burgess Shale (D. Collins, personal communication) and the Sirius Passet fauna will help to make sense of this intriguing group. Anomalocariids may achieve importance, not as a supposedly unique clade of extinct animals, but as a group close to the ancestry of modern arthropods.

The onychophorans, represented today by *Peripatus* of southern tropical forests and its relatives, is now known to have enjoyed considerable success in the Cambrian. These early, marine forms included the hitherto enigmatic *Hallucigenia*⁸³, as well as *Microdictyon*⁸⁴, which before its discovery in the Chengjiang fauna (Fig. 1) was known only from dispersed phosphatic sclerites. The Chengjiang fauna has yielded several other onychophores^{85,86}, and other examples include *Xenusion* from Scandinavia⁸⁷. *Facivermis*⁸⁸ is peculiar because the anterior region bears five pairs of lobopod-like appendages, whereas the rest of the body is smooth. Sclerotization of such an animal, with jointed limbs replacing lobopods, could lead to an animal similar to phosphatized Ordovician fossils from Öland⁸⁹, regar-

ded as primitive pentastomids (still extant as parasitic animals whose postulated position in the crustaceans⁹⁰ has been recently confirmed by molecular evidence⁹¹). There is insufficient information to resolve the phylogenetic relationships between anomalocariids, onychophores and arthropods exactly, but the first group may be a glimpse of what early arthropod-like protostomes were really like. Recent molecular analysis of extant onychophorans supports their place in the arthropods, but questions their primitive status⁹².

Annelids, molluscs and relatives. Further protostome diversification led to a plexus of annelids, molluscs and near relatives. If the scheme of Lake⁷ is followed, then the annelids arose next. In the Burgess Shale there is an undescribed worm (Fig. 4b) with prominent lateral extensions that may be close to the basal annelid stock. The first definitive record of the annelids is as polychaetes⁹³, which are diverse in the Burgess Shale: their absence from the Chengjiang fauna may not be significant because they are present in the slightly younger Sirius Passet fauna. There is general agreement that pogonophorans are related to annelids⁹⁴. The elongate, organic-walled tubes of sabelliditids, abundant in the Vendian and lower Cambrian, may have housed pogonophorans. There are, however, differences in wall ultrastructure between these and modern pogonophoran tubes^{95,96}.

Molecular data suggest that the nemerteans, traditionally placed close to the platyhelminths, are coelomate protostomes, perhaps related to the annelids⁹⁷, but with a distinctive coelom (rhynchocoel surrounding the proboscis). The nemertean fossil record is very meagre, but amiskwiids⁹⁸ (represented by *Amiskwia* from the Burgess Shale) may represent an early stage of their divergence. Molecular evidence likewise places the brachiopods close to the annelids, and the chaetae in both groups are similar⁹⁹. Understanding of the initial radiation of brachiopods is improving, especially with the description of supposedly enigmatic groups^{48,58}. But pitfalls remain; for example, a putative brachiopod-like shell from the lower Cambrian of Irkutsk¹⁰⁰ could be a percussion fracture formed when the drill core was broken open, a telling illustration of the difficulties sometimes incurred in interpreting problematic fossils. Tommotiids, only known from dispersed phosphatic sclerites, have been reconstructed as slug-like animals¹⁰¹. Some sclerites, however, recall brachiopods, and comparison of shell ultrastructure¹⁰² supports the possibility that tommotiids and brachiopods are related⁵⁶.

A relationship between annelids and brachiopods may be difficult to reconcile with data from haemerythrin sequences that indicate a close relationship between priapulids and the inarticulate brachiopod *Lingula*¹⁰³. Nevertheless, current evidence suggests that priapulids (and the closely related kinorhynch and loriciferans, both without a fossil record), are part of the coelomate protostome radiation. Whether Cambrian priapulids^{50,104} will throw light on early protostome relationships is difficult to judge. Fossils called palaeoscolecidans, known to bear phosphatic sclerites¹⁰⁵, may be of interest here: specimens from the Sirius Passet fauna display what look like spiny snouts similar to those that distinguish priapulids, suggestive of a close relationship. Conversely, rows of phosphatic sclerites similar to those of palaeoscolecidans have been found in a Burgess Shale priapulid¹⁰⁴ (Fig. 4d). Of potential significance also is the Chengjiang priapulid *Cricocosmia*¹⁰⁶, which has two rows of convex shell-like sclerites on its trunk.

Molluscs may have evolved from a flatworm-like ancestor independently of the annelids⁴, but molecular evidence¹⁰ supports the possibility of an annelid link. The fossil record is silent on this transition, but the earlier stages of mollusc evolution are becoming clear. Cardinal evidence comes from the halkieriids⁶³ (Fig. 4c), which had an articulated armour of sclerites together with prominent anterior and posterior shells. A number of mollusc-like shells are known from the Cambrian and it is likely that some derive from halkieriid or related scleritomes.

*Triplicatella*⁴⁸, from South Australia and possibly southern Siberia¹⁰⁷, very probably comes from the scleritome of the halkieriid. *Thambetolepis*. Prominent folds on the shell margin of *Triplicatella* may mark sites of exhalant and inhalant water currents⁴⁸, perhaps connected with gills similar to molluscan ctenidia.

In turn, similarity of sclerite structure suggests a link between halkieriids and the Coeloscleritophora⁴⁸, a diverse Cambrian group that includes siphonochitids, sachtids and chancelloriids. The first of these probably had a scleritome similar to halkieriids, with at least one shell and two types of sclerite¹⁰⁸. Chancelloriid sclerite structure does not support their earlier assignment to sponges, but overall body shape is consistent with a sessile habit. Chancelloriid sclerites were evidently embedded in a resistant cuticle (Hou Xianguang, personal communication). Sachtids may be intermediate in form between chancelloriids and halkieriids⁴⁸.

Trace fossils

Even if no soft-bodied fossils had been preserved in the Cambrian, their adaptive radiations would be evident from the record of trace fossils^{58,60}. Although some traces, such as scratch marks, can be attributed to arthropods (or perhaps the related anomalocariids), in general tying traces to their makers is difficult. Trace fossils have consequently played little part in discussions of metazoan phylogeny, but they may nevertheless contribute useful information. Although many kinds of trace fossil are known from a long geological time span, some are confined to the Cambrian and could record extinct body plans, as well as details of early metazoan activities such as locomotion and feeding. For example, the giant trace *Climactichnites* from the Upper Cambrian of North America is believed to have been constructed by an otherwise unknown Metazoan of novel appearance¹⁰⁹.

Phyla and the role of problematic taxa

Schemes of metazoan phylogeny based on molecular evidence but which have been broadened to include information from fossils have been presented before^{110,111}. I have taken it a stage further with the inclusion of many groups usually seen as problematic, linking them with more familiar body plans. But this is not meant to belittle the magnitude of the adaptive radiations that took place in the Vendian and Cambrian periods. Within a period of about 20 Myr (taking the Ediacaran faunas as ~560 Myr old and the base of the Cambrian as ~540 Myr old¹¹²), the oceans changed from habitats housing a rich but effectively microscopic biota¹³, to one teeming with macroscopic animals engaged in a wide range of ecologies and presumably showing a degree of behavioural sophistication. Is it realistic to talk of a multiplicity of body plans in the Cambrian, far exceeding that of the present day¹¹³? At the other extreme, the argument that extant phyla maintain their identity back to the Cambrian¹¹⁴ offers no more than a tautology. Such phyla so persist because the only way they can be recognized is by reference to themselves. Consider the potential disagreements that surround the interpretation of various Cambrian fossils: are the pseudo-brachiopods⁴⁸ 'truly' brachiopods? Are the anomalocariids⁸² 'really' arthropods? Are hyoliths or stenotheoids 'actually' molluscs? Definitions of major groups are less secure than sometimes imagined.

I argue that the supposed problematic taxa, now in imminent danger of elevation to a classic status as evolutionary enigmas¹¹³, hold the key to understanding many aspects of early metazoan evolution. This view contrasts with that of a 'pool' of generalized coelomate ancestors from which at least the main phyla of protostomes were derived^{114,115}. In such models the problematic taxa are further and separate derivatives, useful for documenting the range of metazoan morphospace but irrelevant for establishing relationships at the base of metazoan evolution.

Conclusion

Future advances will depend largely on molecular biologists and palaeontologists, although no data-source should be neglected¹¹⁶. The former need to tackle new gene sequences and extend the database. It is necessary also to enlarge our understanding of developmental mechanisms, and there is growing interest in the widespread occurrence of at least some regulatory genes in disparate phyla^{117,118}. What is equally important to know, however, is how these genes have either been co-opted or have changed their function, not only among metazoan phyla but in their protistan ancestors.

What can palaeontologists contribute? The discovery of Cambrian faunas similar to that of the Burgess Shale is likely⁶¹. Further preparation of collections of calcareous and phosphatic shelly Cambrian fossils may well produce dividends, and such disarticulated remains that are etched out of the rock should be held up against articulated scleritomes, either actual or hypothesized. The diversification of trace fossils still lacks a biological context, and there is an urgent need to test whether at least some Ediacaran fossils could be assigned to the Vendobionta, rather than to Metazoa³⁹.

And yet, at the heart of the matter, a coherent explanation for the origin and scope of the early metazoan radiations is still missing⁵⁸. Family trees based on molecular data can be festooned

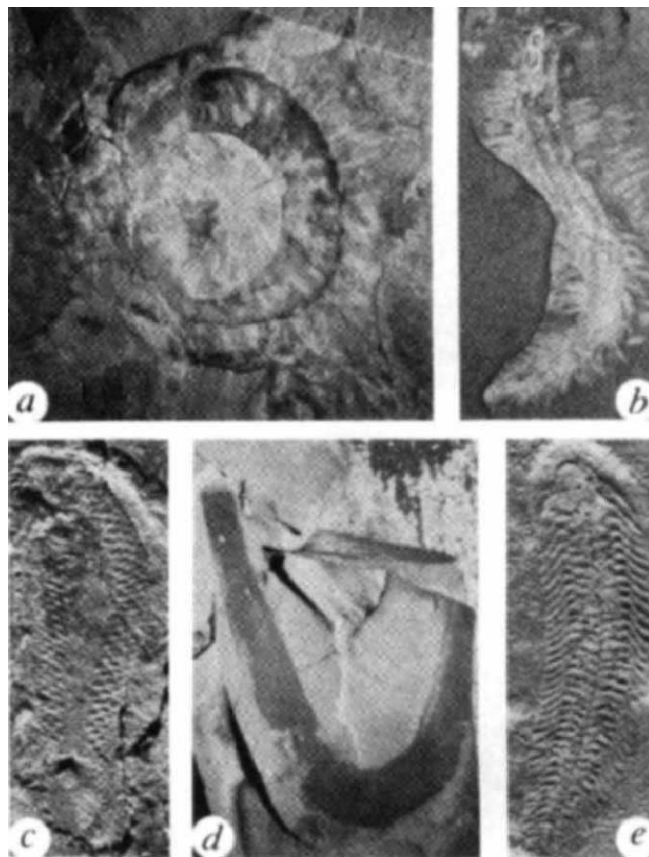


FIG. 4 Burgess Shale-type fossils (a–d) and a possible stem arthropod (e). a, *Eldonia ludwigi*, an early deuterostome that may show features of pre-echinoderms; magnification, 1.1×. Specimen from Middle Cambrian (Burgess Shale), British Columbia. b, Undescribed worm from Middle Cambrian (Burgess Shale), British Columbia, possibly representing an early annelid; 3.2×. c, Articulated halkieriid from the Lower Cambrian (Buen Formation) of the Sirius Passet fauna, north Greenland; 1.5×. Halkieriids appear to represent a very early stage of mollusc evolution. d, The priapulid worm *Louisella pedunculata* from the Middle Cambrian (Burgess Shale), British Columbia; 0.85×. The trunk bears rows of sclerites, closely similar in arrangement to those of palaeoscolecidans. e, *Spriggina floundersi*, with segmented body and cephalic shield, a possible member of the arthropod stem group from the Vendian Pound Subgroup, south Australia; 2.3×.

with fossils but this is perhaps rather premature. Instead, we should be asking how the diversity of body plans so evident in the Vendian and Cambrian affected the shape of things to come. Evolutionary innovations such as nerve tracts, seriation, mesoderm, spacious body cavities and circulatory systems, all implied by the fossils already available, must have had profound phylogenetic consequences. Again, the causes of this diversification still remain a mystery, although changes in the concentration of atmospheric oxygen¹¹⁹, trophic resources, and ecological response, especially to predation, may have all played a part. Certainly the acquisition of hard parts as a deterrent to

predators is a compelling hypothesis¹²⁰. Increasing evidence for major changes in the environment during the Vendian and Cambrian¹²¹ also require further assessment. The once stagnant field of metazoan phylogeny is being rejuvenated by new discoveries that promise to call a wide range of scientific disciplines. 'Metazoan Phylogeny' is exciting to play, and we may, at last, be on the verge of working out some of the rules of the game. □

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The gene involved in X-linked agammaglobulinaemia is a member of the *src* family of protein-tyrosine kinases

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X-linked agammaglobulinaemia (XLA) is a human immunodeficiency caused by failure of pre-B cells in the bone marrow to develop into circulating mature B cells. A novel gene has been isolated which maps to the XLA locus, is expressed in B cells, and shows mutations in families with the disorder. The gene is a member of the *src* family of proto-oncogenes which encode protein-tyrosine kinases. This is, to our knowledge, the first evidence that mutations in a *src*-related gene are involved in human genetic disease.

X-LINKED agammaglobulinaemia (XLA; Bruton type; MIM 30030; gene symbol AGMX1) was the first described immunoglobulin deficiency¹. Affected males lack circulating mature B cells and serum immunoglobulins of all isotypes, and suffer recurrent bacterial infections². The infections, particularly bacterial meningitis and pneumonia, are life-threatening at an early age and require the use of antibiotic and immunoglobulin replacement therapy. Affected males have a normal number of pre-B cells in their bone marrow, suggesting that the XLA defect resides in the pathway of B-cell development³. This defect is specific to the B-cell lineage because other lymphocyte populations appear to be normal. Heterozygous females appear immunologically normal, as a result of selection against B cells having the mutant X chromosome active. Such 'skewing' of X chromosome activity in the B-cell population reliably identifies the carrier status^{4,5}. The isolation of the XLA gene would aid in understanding the disorder, its role in B-cell development, and allow accurate carrier and prenatal diagnosis directly by mutation analysis. Because the disorder is specific to B cells, it may be a candidate for somatic gene therapy.

As with XLA, most human genetic disorders arise from mutations in genes that encode currently unknown proteins. The identification of such genes may be achieved by positional cloning (for review, see ref. 6). Once extensive mapping information becomes available, candidate genes can be isolated in the region surrounding the disease locus and used to identify mutations in patients, demonstrating a direct relationship between a gene and a disease phenotype. This task is being made increasingly efficient through the isolation of large tracts of the human genome in cloned form^{7,8}, the development of novel strategies to detect genes⁹⁻¹², and by using efficient methods of mutation detection^{13-15,16}.

We report the isolation of a gene defective in XLA. A yeast artificial chromosome (YAC) clone was used for direct selection of candidate genes as complementary DNAs^{11,12}. One candidate was found to be a novel member of the *src* family of genes, encoding a protein-tyrosine kinase expressed in B cells. Deletions and point mutations of this gene were detected in eight unrelated XLA patients, strongly suggesting that it is directly

involved in the disease and, therefore, in the process of B-cell development.

Cloning strategy

The XLA locus has been mapped to the region Xq21.3-Xq22 (refs 17-21 and R. Lovering *et al.*, manuscript in preparation). The consensus order of loci in the region was established as cen-DXS3-(XLA, DXS178)-DXS94-DXS17-tel, with the polymorphic marker DXS178 showing no recombination with the disease (refs 20, 21 and R. Lovering *et al.*, manuscript in preparation), and DXS3 and DXS94 lying approximately 10 centimorgans (cM) apart (Fig. 1a). Recently, the identification of recombinants between XLA and the flanking markers DXS442 and DXS101 resulted in the further localization of XLA to within a 2 cM interval (R. Lovering *et al.*, manuscript in preparation).

Long-range restriction maps have been constructed in Xq21.3-Xq22 (refs 22, 23 and D.V. *et al.*, manuscript in preparation). The order of 12 markers flanking the XLA locus was established, and DXS178 was shown to lie 70-140 kilobases (kb) distal to GLA (the α -galactosidase A gene) and about 500 kb proximal to DXS101 (ref. 22 and D.V. *et al.*, manuscript in preparation; Fig. 1a). These studies also identified a number of putative CpG islands²⁴ in the region around DXS178 (ref. 22, D.V. *et al.*, manuscript in preparation), indicative of the presence of genes. The entire region extending from DXS442 to beyond DXS17 was isolated in a contiguous set of 68 overlapping YAC clones (that is, a contig) spanning 6.5 megabases (Mb) (D.V., manuscript in preparation). These studies formed the basis for a systematic search for the gene involved in XLA.

A 640 kb YAC clone y178-3 containing the GLA and DXS178 loci was chosen from the contig. The artificial chromosome was gel-purified and used as a hybridization probe to identify 48 cosmid clones in a library constructed using DNA from cells of karyotype 49,XXXXX (ref. 25). Positive cosmids were mapped into intervals by hybridization to other YACs and isolated ends of YAC clones from the contig, and to the GLA and DXS178 probes (ref. 26 and I.V. *et al.*, manuscript in preparation).

To identify transcribed sequences within y178-3, we modified the technique of cDNA selection^{11,12} (see Fig. 1 legend for details) which results in the enrichment of region-specific cDNAs in a cDNA population in the order of 10²- to 10⁴-fold²⁷.

[†] D.V. and I.V. contributed equally to this study.

DNA from YAC y178-3 was gel-purified, immobilized on nylon filters, and the filters were hybridized to inserts amplified from a total cDNA library. Previous reports on B-cell differentiation in XLA (refs 2, 28) suggested to us that the disease gene may be expressed throughout B lymphocyte development. Therefore, a single round of selection was done using the cDNA library BL-29 (ref. 29) derived from an Epstein-Barr virus (EBV)-

positive Burkitt's lymphoma cell line. The bound material was recovered by denaturation, reamplified and either used as a probe (see below) or subcloned and gridded in high-density arrays³⁰. Screening of the enriched and original BL-29 cDNA libraries with a probe for GLA indicated enrichment by a factor of 300 (data not shown).

The pool of enriched BL-29 polymerase chain reaction (PCR)

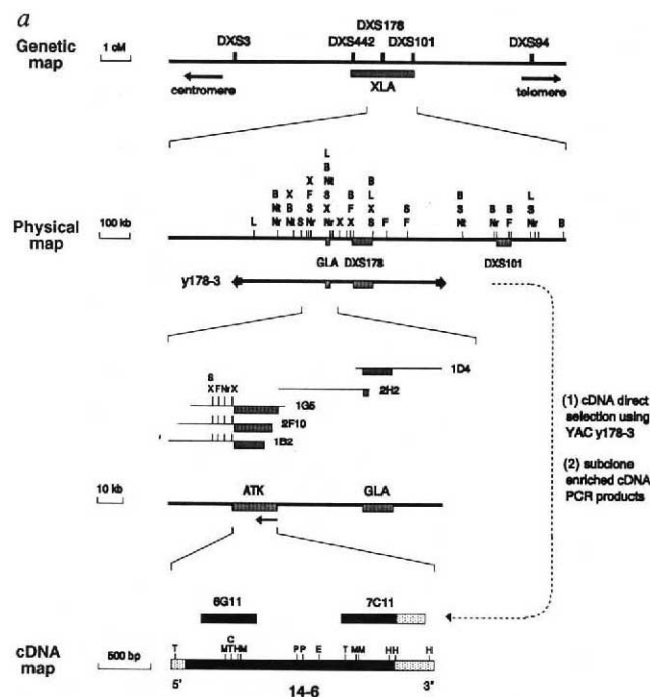
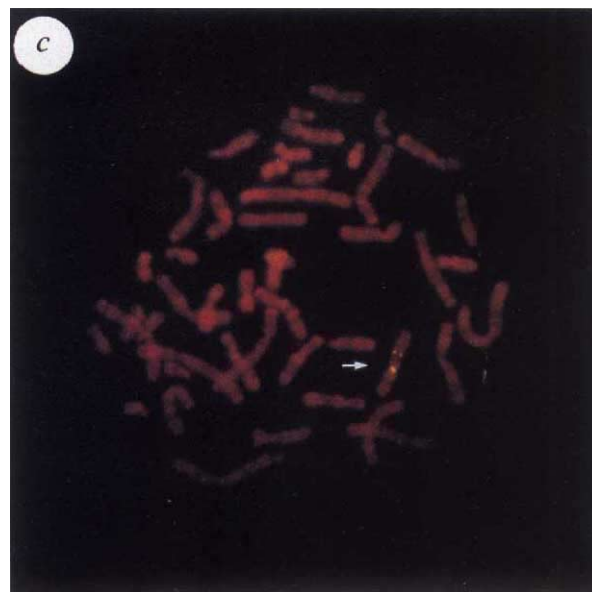
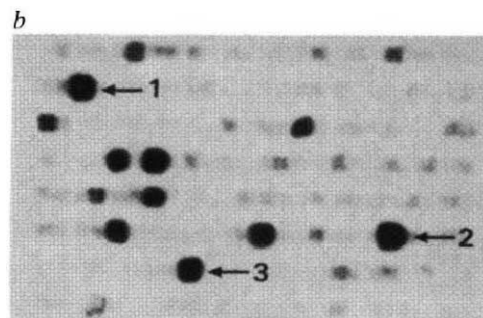


FIG. 1 *a*, Positional cloning of the *atk* gene involved in X-linked agammaglobulinaemia (XLA). The gene defective in XLA was localized to a 2-cM interval in Xq21.3-Xq22 (R. Lovering *et al.*, manuscript in preparation) which is shown on the genetic map. A long-range restriction map around the DXS178 locus is shown (D.V. *et al.*, manuscript in preparation and ref. 22). The location of the GLA, DXS178 and DXS101 loci are shown as the shaded rectangles. The 640-kb YAC clone y178-3 contains both the GLA and DXS178 loci. Among the 48 cosmid clones isolated with this YAC, five are shown that form a contig flanking the GLA locus. Two of these cosmids (1D4 and 2H2) contain at least part of the α -galactosidase A gene and the remaining three (1G5, 2F10 and 1B2) contain the ATK locus. The ATK locus encompasses a 20-kb genomic region located 50–70 kb centromeric of GLA. YAC clone y178-3 was used in cDNA direct selection experiments with the BL-29 cDNA library. Two of the resultant subcloned cDNAs, 7C11 and 6G11, mapped, along with 23 other subcloned products, to the ATK locus. The full-length cDNA represented by clone 14-6 was subsequently isolated from the original BL-29 cDNA library. Coding regions found in cDNAs 14-6, 7C11 and 6G11 are shown as dark shaded regions. The arrow below the shaded rectangle representing the ATK locus shows the direction of transcription is toward the centromere, although the 14-6 cDNA is shown 5'→3' from left to right (by convention). The restriction sites shown on the long-range restriction map and the cDNA map are sites for *Bss*III (B), *Clal* (C), *Eco*RI (E), *Hind*III (H), *Mlu*I (L), *Msp*I (M), *Not*I (N), *Nru*I (Nr), *Pst*I (P), *Sal*I (S), *Sfi*I (F), *Taq*I (T) and *Xho*I (X). *b*, Results of hybridizing the pool of enriched BL-29 PCR products to a high-density array of cosmids isolated with y178-3. The arrows 1–3 show the positions of cosmids 1B2, 2F10 and 1G5, respectively. *c*, Assignment of cosmid 2F10 to the border of Xq21.3-Xq22 by fluorescence *in situ* hybridization. The 2F10 signal is shown relative to the X-chromosome centromere (indicated by the pSV2X5 alphoid repeat marker).

METHODS. YAC clone y178-3 was isolated from the ICRF YAC library⁵⁶ by hybridization³⁰. This YAC was gel-purified, and used as a hybridization probe to screen the cosmid library constructed from cells with karyotype 49,XXXXX as previously described²⁵. Positive cosmids were ordered in intervals and subsequently into contigs^{25,26}. PCR amplification of cDNA inserts of the BL-29 library was with M13 forward and reverse primers 5'-GTAAAC-GACGGCCAGT-3' and 5'-AACAGCTATGACCATG-3', respectively. About 10–50 ng of purified YAC DNA was immobilized onto 6 mm² nylon filters. These filters were prehybridized in a volume of 80 μ l containing 5 $\times$ SSC, 5 $\times$



Denhardt's, 0.5% SDS, 1 mg ml⁻¹ sheared human placental DNA (Sigma), and 0.025 mg ml⁻¹ total yeast DNA (strain AB1380). Filters were then briefly rinsed in 5 $\times$ SSC, 0.1% SDS at room temperature and transferred to fresh hybridization solution containing amplified cDNA (pre-annealed with human placental DNA for 90 min) at a final concentration of 10 μ g ml⁻¹. Hybridization was done at 65 °C for 24 h under mineral oil. Filters were washed twice in 2 $\times$ SSC, 0.1% SDS at room temperature, three times at 65 °C, 0.2 $\times$ SSC, 0.1% SDS, and four times at 65 °C, 0.1 $\times$ SSC, 0.1% SDS. Filters were added directly to a PCR reaction and amplification was with the M13 primers (forward and reverse), and then with the nested set of primers T3 (5'-ATTAACCTCACTAAAG-3') and T7 (5'-AATACGACTCACTATAG-3'). Only a single round of selection was done. The enriched cDNA PCR products were subcloned into a T-tailed⁵⁷ version of pBluescript SK (+/-) and gridded as high-density arrays³⁰. Cosmids containing transcripts were identified by hybridization of the pooled, enriched cDNA PCR products to cosmids positive for y178-3. The cDNA PCR products and gel-purified cosmid fragments were pre-annealed with human placental DNA⁵⁸ before their use in hybridization experiments. Subcloned cDNA inserts were amplified with either the M13 primers or with T3 and T7. cDNA clones were isolated from the original BL-29 library by hybridizing 7C11 and 6G11 to plaque lifts as previously described⁵⁹. Inserts from purified plaques were also PCR-amplified using T3 and T7 primers. *In situ* hybridization was done as follows: biotin-labelled cosmid 2F10 and pSV2X5 were pre-annealed with Cot-1 DNA and then hybridized to metaphase spreads of cultured lymphocytes from a normal male. Signal detection was by alternate layers of avidin-FITC and biotinylated anti-avidin. Chromosomal banding was by late BrdU incorporation, photolysis in the presence of Hoescht 33258 and staining with propidium iodide.

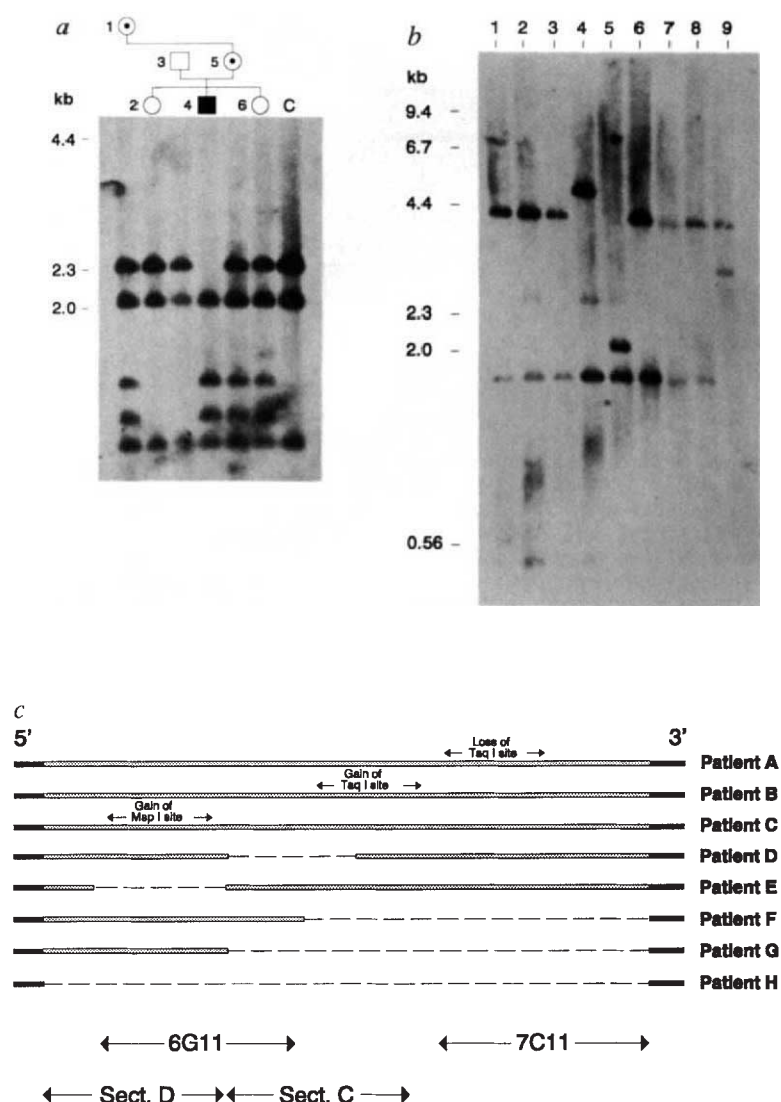
products was used as a probe to hybridize to filters of the cosmid clones that had previously been isolated with YAC clone y178-3. Thirteen of the 48 cosmids gave a strong positive signal (Fig. 1b). Of the 1,536 sub-cloned cDNAs, 104 were detected by hybridization to restriction fragments of these cosmids, and arranged into 12 mapping groups (ref. 26 and I.V., manuscript in preparation). Representative cDNAs from each group were used to screen Southern blots from XLA patients. cDNAs from two of these groups (cDNAs 7C11 and 6G11; Fig. 1a) detected DNA rearrangements in XLA families (see below). Three cosmids spanning these two cDNA groups (1B2, 2F10 and 1G5, highlighted in Fig. 1a and b) hybridized to a total of 25 sub-cloned cDNAs, all of which were localized to a 20 kb region about 50–70 kb centromeric of the α -galactosidase A gene. The cDNAs 7C11 and 6G11 were used to screen the original BL-29 cDNA library to isolate longer, possibly full-length clones. Clones isolated from the original cDNA library, the longest of which was the 2.6 kb cDNA 14-6 (Fig. 1a), hybridized under high stringency to all 25 of the subcloned cDNAs originating from the 20-kb genomic region of cosmids 1B2, 2F10 and 1G5. Therefore, these 25 subcloned cDNAs were derived from a single transcript. On the basis of the abundance of this sequence in the original BL-29 cDNA library (3 in 100,000 clones), about 500-fold enrichment of this particular transcript was achieved. This sequence was localized to both the YAC contig and the long-range restriction map of the region. In addition, *in situ*

hybridization of cosmid 2F10 to metaphase human chromosome spreads demonstrated a unique signal at the Xq21.3–Xq22 boundary (Fig. 1c).

DNA rearrangements in XLA families

Southern analysis of XLA patients from 33 unrelated families and 150 normal X chromosomes was done with *TaqI* and *MspI* using cDNA probes 7C11 and 6G11 and a 10-kb *SmaI* genomic restriction fragment from cosmid 2F10. Restriction pattern abnormalities were detected in eight XLA families, as summarized in Fig. 2. No variation was observed in the hybridization patterns of the normals. Restriction fragment alterations in the DNA of patients A and B were found using *TaqI*, but not *MspI*, and were consistent with the loss and gain of a *TaqI* site, respectively (later confirmed by sequence analysis as described below). These alterations were shown to segregate with all family members known to have the mutant XLA locus in their respective pedigrees (see Fig. 2a for pedigree analysis of patient B). Figure 2b shows an example of the abnormal restriction fragment patterns observed in patients C, E and G. The abnormality in patient C was detected only with *MspI* Southern blots. Patients D–H were shown to have deletions of genomic DNA containing 7C11 and 6G11 coding regions, as depicted in Fig. 2c. Further analysis of the deletions using additional sections of the 5' half of the cDNA clone 14-6 (see legend to Fig. 2c for details) confirmed that patients D and E both contain normal restriction

FIG. 2 Identification of restriction fragment alterations in XLA patients. *a*, The 10 kb *SmaI* restriction fragment from cosmid 2F10 detected novel 1.0 kb and 1.3 kb *TaqI* restriction fragments, instead of the normal 2.3 kb fragment, in patient B (lane 4). This alteration was observed in the patient's mother and maternal grandmother (lanes 5 and 1, respectively), who are obligate carriers as both of them have had affected sons. The abnormal hybridization pattern was also seen in one of patient B's sisters (lane 6), for whom carrier status had not previously been determined. Lanes 2, 3 and C (unrelated normal control) show the normal restriction patterns. Size markers are shown in kilobases. *b*, Abnormal *MspI* fragment patterns were detected in patients E, C and G (lanes 4, 5 and 9, respectively) and also in patient D (not shown) using the partial cDNA 6G11. Abnormal *TaqI* fragments were also detected in DNA from patients D, E and G using 6G11 (not shown), suggesting that these patients had DNA rearrangements. Size markers are shown in kilobases. *c*, Schematic representation of the DNA alterations found in eight XLA patients. The lightly shaded region is the genomic region of DNA encompassing the *atk* gene. Dashed lines show the regions of genomic DNA that are deleted in five XLA patients. The approximate regions of genomic DNA containing coding sequence found in 6G11 and 7C11 are indicated. The cDNA probes corresponding to sections D and C (nucleotides 1–652, and 653–1,453, respectively in Fig. 3a) are also shown. No signal was observed when DNA from patients F, G and H was hybridized to cDNA 7C11 (results not shown) indicative of DNA deletions. We confirmed that this result was not due to any differences in the amounts of DNA used in each lane of the Southern blot, because a second Xq22 marker detected restriction fragments in all samples. Southern analysis of selected samples using cDNA sections D and C showed that the deletions in patients D and E are entirely intragenic. Section D (which represents the 5' end of the *atk* cDNA sequence) detected 0.3, 1.0, 1.1, 1.5 and 4.3 kb *MspI* fragments in normal genomic DNA; in patient E no 4.3 or 1.5 kb *MspI* fragments were observed, while a novel 5 kb band was seen. cDNA 6G11 (which does not extend to the 5' terminus of the *atk* gene) hybridized to 0.3, 0.5, 1.7, 2.5 and 4.3 kb *MspI* fragments in normal individuals and also detected the abnormality in patient E. Section C detected no *MspI* abnormalities in patient E. Section C detected 0.5, 0.8, 1.7 and 2.5 kb *MspI* fragments in normal DNA; the 1.7 kb fragment was absent in patient D, but a novel 0.6 kb band was observed. Section D did not detect any abnormalities in patient D.



fragments derived from the 5' end of the gene in addition to those detected near the 3' end with probe 7C11. The deletions in these two patients are therefore entirely intragenic, confirming the involvement of this gene in XLA.

A novel protein-tyrosine kinase gene

The complete nucleotide sequence of the longest cDNA clone 14-6, isolated from the original BL-29 library, was determined and is shown in Fig. 3a. The cDNA comprises 2,560 nucleotides and has an open reading frame encoding a 659-amino-acid polypeptide beginning with the initiation codon at nucleotide position 133. The reading frame is closed 15 codons upstream of this start site. Two alternative initiation codons are located at nucleotide positions 397 and 619 within the same open reading frame as the first; initiation of translation from these two start signals would result in peptide chains of 571 and 497 amino acids, respectively. All three of these start positions agree well with Kozak's consensus sequence³¹. In the absence of the protein product encoded by this transcript, it is not yet possible to predict which start signal is most favoured. The consensus polyadenylation signal AATAAA is present at position 2,524, 20 nucleotides upstream from the poly(A)⁺ tail.

The sequence of cDNA 14-6 is most similar to that of the *src* family of proto- (that is, cellular) oncogenes. Members of the *src* gene family, characterized by extensive homology to the kinase domain of the transforming gene of the Rous sarcoma virus (*v-src*), encode intracellular (non-receptor) protein-tyrosine kinases. These proteins are usually bound to the plasma membrane by myristylation and/or direct attachment to membrane proteins^{32,33}. It is thought that members of the *src* family play important roles in the cellular signalling pathways that regulate cell proliferation and differentiation. Protein-tyrosine kinases function as central switching mechanisms in these pathways by catalysing the phosphorylation of tyrosine residues in specific target proteins (for review, see ref. 34). Because of the nature of their role in cellular development, genes encoding proteins with tyrosine kinase activity form a large proportion of known proto-oncogenes.

Across a stretch of about 1,200 bp of its open reading frame, the DNA sequence of cDNA 14-6 shows a high degree of similarity with two members of this gene family: the murine *tec* gene (64% DNA sequence identity)³⁵ and the *Drosophila src28C* gene (58%)³⁶. On the basis of this extensive similarity and our data presented below, we have called the gene represented by cDNA 14-6, *atk* (for agammaglobulinaemia tyrosine kinase). At the amino-acid level, *atk* shows highest similarity to the murine *tec*, and to the human *src* and *yes* gene products (Fig. 3b). It possesses a putative kinase domain of 252 residues (amino-acid positions 408 to 659) which shows 64% amino-acid identity with the *tec* protein. This domain contains the ATP-binding site at positions 408 to 430, and includes Lys 430, the residue equivalent to the highly conserved Lys 295 of *v-src*. This residue, present in all functional protein-tyrosine kinases, is essential for catalytic activity³⁷. Also within the predicted kinase domain of *atk* is a substrate-specific domain (amino acids 519–567) that determines the specific phosphorylation of tyrosine residues. Within this region, Tyr 551 of *atk* is presumed to be an important auto-regulatory site by analogy with the role of the corresponding Tyr 416 of *v-src* (ref. 38). The putative kinase domain of *atk* does not, however, have the equivalent residue corresponding to a second auto-regulatory site at Tyr 527 found in many cellular *src* proteins. The absence of this residue, as well as the presence of Leu 516 (found in the *atk* amino-acid sequence possibly as Leu 652) are features of *src* proteins that are capable of inducing cellular transformation^{39,40}. *atk* also possesses two *src* regulatory domains corresponding to SH2 and SH3 at positions 281–377 and 219–269, respectively (for a review, see refs 41 and 42). The SH2 domain is thought to bind phosphorylated tyrosines in protein substrates⁴³, as well as providing a mechanism for auto-regulating the catalytic activity of *src*

proteins; the SH3 domain may bind cytoskeletal components³⁴. As is typical of protein-tyrosine kinases, the putative amino-terminus of *atk* shows little homology with its most closely related *src* counterparts. Therefore, this region may confer some degree of specialization to the protein, in terms of cellular location and/or tissue specificity. Three putative myristylation sites can be recognized at positions 50, 150 and at 164 of *atk*. The third of these alternatives might indicate the initiation of translation is at Met 163 (nucleotide position 619), because glycine at position 2 is a myristylation site found in *src* proteins⁴⁴.

Tissue specificity of the *atk* transcript

To investigate the expression pattern of this transcript, we did northern blot analysis of RNA from various tissues using the cDNA clones 6G11 and 14-6 as hybridization probes. A 2.6-kb transcript was detected in B cells, adult lung and pancreas (Fig. 4), indicating that the 2.6 kb cDNA 14-6 represents a virtually full-length transcript. Longer exposures revealed a weakly hybridizing transcript of similar size in adult heart, brain, liver, kidney and skeletal muscle, as well as a 6-kb transcript in lung and pancreas (Fig. 4a). The larger transcript may correspond to either an unprocessed version of the 2.6-kb species or a distinct RNA species that bears some homology to the *atk* cDNA. Northern analysis of RNAs derived from lymphoid lineages showed that the 2.6 kb *atk* messenger RNA was expressed in the B-cell line NAD, and also in B cells of two patients with chronic lymphocytic leukaemia (B-CLL 1, B-CLL 2), but not in T cells (T-CLL) or a T-cell line (Jurkat) (Fig. 4b). These results further support the involvement of *atk* in XLA.

Point mutations in the *atk* kinase domain

Reverse-transcriptase (RT)-based PCR of total lymphocyte RNA has been shown to amplify specific coding sequences regardless of their primary tissue of expression^{16,45}. Using this approach, we amplified sections of the *atk* cDNA sequence from patients A and B. *TaqI* restriction site changes co-segregate with the disease in the families of both these patients (see above). A *TaqI* restriction site was found to be absent from the DNA of patient A, whereas patient B had gained a site for this restriction enzyme. A 225-bp PCR product amplified from cDNA of patient A using oligonucleotide primers B5 and A3 (Fig. 5a) was digested with *TaqI*. This amplification product did not cleave into the expected 107 bp and 118 bp fragments in the patient, indicating that the loss of the *TaqI* site was in the coding sequence (at cDNA position 1,704). The alteration was also seen in the 225-bp amplification product derived from lymphocyte RNA of the obligate carrier mother (Fig. 5b). A similar approach was used to determine that the location of the new *TaqI* site in patient B was in the cDNA sequence, as digestion of a 518-bp PCR product (generated with oligonucleotide primers A5i and A3; Fig. 5a) with *TaqI* resulted in the appearance of three *TaqI* fragments of 107 bp, 125 bp and 286 bp, instead of the expected normal pattern of 107 bp and 411 bp fragments only (Fig. 5b). The aberrant pattern was also observed in patient B's maternal grandmother (an obligate XLA carrier).

Direct DNA sequencing⁴⁶ of the relevant amplification products in both patients was used to characterize each mutation. Patient A had a G→A transition at position 1,706, whereas patient B had an A→G transition at position 1,420 (Fig. 5c). On the basis of the proposed amino-acid sequence encoded by *atk*, these single base changes would result in an Arg→Gln change at amino-acid position 525 in patient A, and a Lys→Glu change at residue 430 in patient B (Fig. 5c). Both of these non-conserved amino-acid substitutions would have highly detrimental effects on the catalytic function of the putative protein-tyrosine kinase. The loss of the conserved Arg 525 in patient A could prevent substrate recognition because this residue is thought to be important in the substrate-specific domain⁴⁷. Substitution of Lys 430 in patient B (equivalent to

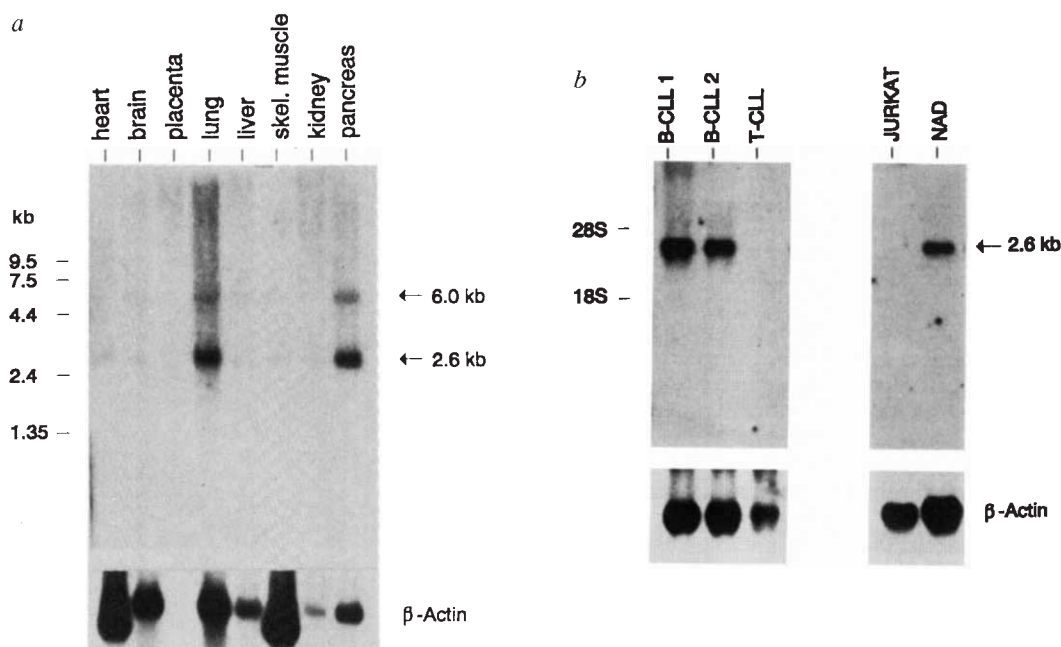
FIG. 3 *a*, The DNA sequence of *atk* found in the 2,560 bp cDNA clone 14-6. The three-letter abbreviations for the 659 amino acids encoded in the open reading frame beginning at nucleotide position 133 are also shown. The three potential start sites and the polyadenylation signal are underlined (see text for further description). *b*, Comparisons of the predicted amino-acid sequence of *atk* with that of closely related proteins of the *src* family. Residues found in *atk* that are identical to those found in murine *tec*, human *src* or *yes* are shaded. The putative SH2 and SH3 domains, the ATP-binding

site, and the substrate-specific domain described in the text are boxed. Lys 430 and Tyr 551 (=Lys 295 and Tyr 416 of *v-src*) are also highlighted. Tyr 527 found in *src* and *yes*, but not in *atk* and *tec*, is shown in brackets. METHODS. cDNA 14-6 was sequenced using the dideoxy chain termination method⁶⁰. Complete sequence for both strands was accomplished by primer-walking. Initial searches for homology between *atk* and other known gene sequences was through the EMBL nucleotide sequence database. Protein alignment was by visual inspection.

<i>a</i>	CGTATGTCTCCAGGGCCAGTGTCTGCTGCGATCGAGTCCACCTTCCAAAGCTCTGGCATCTCAATGATCTGGGAAGCTACCTGCATTAAAGTCAGGACTGAGCACACAGGTGAATCCAGAAAGAAGAGCT																														1	5													
																															Met	Ala	Ala	Val	Ile										
																															ATG	GCC	GCA	GTG	ATT										
	10	15	20	25	30	35	40	45																																					
148	Leu	Glu	Ser	Ile	Phe	Leu	Lys	Arg	Ser	Gln	Gln	Lys	Lys	Lys	Thr	Ser	Pro	Leu	Asn	Phe	Lys	Lys	Arg	Leu	Phe	Leu	Leu	Thr	Val	His	Lys	Leu	Ser	Tyr	Tyr	Glu	Tyr	Asp	Phe	Glu					
	CTG	GAG	AGC	ATC	TTT	CTG	AAG	CGA	TCC	CAA	CAG	AAA	AAG	AAA	ACA	TCA	CCT	CTA	AAC	TTC	AAG	AAG	CGC	CTG	TTT	CTC	TTG	ACC	GTG	CAC	AAA	CTC	TCC	TAC	TAT	GAG	TAT	GAC	TTT	GAA					
	50	55	60	65	70	75	80	85																																					
268	Arg	Gly	Arg	Arg	Gly	Ser	Lys	Lys	Gly	Ser	Ile	Asp	Val	Glu	Lys	Ile	Thr	Cys	Val	Glu	Thr	Val	Val	Pro	Glu	Lys	Asn	Pro	Pro	Glu	Arg	Gln	Ile	Pro	Arg	Arg	Gly	Glu	Glu						
	CGT	GGG	AGA	AGA	GGC	AGT	AAG	AAG	GGT	TCA	ATA	GAT	GTT	GAG	AAG	ATC	ACT	TGT	GTT	GAA	ACA	GTG	GTT	CCT	GAA	AAA	AAT	CCT	CCT	CCA	GAA	AGA	CAG	ATT	CCG	AGA	AGA	GGT	GAA	GAG					
	90	95	100	105	110	115	120	125																																					
388	Ser	Ser	Glu	Met	Glu	Gln	Ile	Ser	Ile	Ile	Glu	Arg	Phe	Pro	Tyr	Pro	Phe	Gln	Val	Val	Tyr	Asp	Glu	Gly	Pro	Leu	Tyr	Val	Phe	Ser	Pro	Thr	Glu	Glu	Leu	Arg	Lys	Arg	Trp	Ile					
	TCC	AGT	GAA	ATG	GAG	CAA	ATT	TCA	ATC	ATT	GAA	AGG	TTC	CCT	TAT	CCC	TTC	CAG	GTT	GTA	TAT	GAT	GAA	GGG	CCT	CTC	TAC	GTG	TTC	TCC	CCA	ACT	GAA	GAA	CTA	AGG	AAG	CGG	TGG	ATT					
	130	135	140	145	150	155	160	165																																					
508	His	Gln	Leu	Lys	Asn	Val	Ile	Arg	Tyr	Asn	Ser	Asp	Leu	Val	Gln	Lys	Tyr	His	Pro	Cys	Phe	Trp	Ile	Asp	Gly	Gln	Tyr	Leu	Cys	Cys	Ser	Gln	Thr	Ala	Lys	Asn	Ala	Met	Gly	Cys					
	CAC	CAG	CTC	AAA	AAC	GTA	ATC	CGG	TAC	AAC	AGT	GAT	CTG	GTT	CAG	AAA	TAT	CAC	CCT	TGC	TTC	TGG	ATC	GAT	GGG	CAG	TAT	CTC	TGC	TGC	TCT	CAG	ACA	GCC	AAA	AAT	GCT	ATG	GGC	TGC					
	170	175	180	185	190	195	200	205																																					
628	Gln	Ile	Leu	Glu	Asn	Arg	Asn	Gly	Ser	Leu	Lys	Pro	Gly	Ser	Ser	His	Arg	Lys	Thr	Lys	Lys	Pro	Leu	Pro	Pro	Thr	Pro	Glu	Glu	Asp	Gln	Ile	Leu	Lys	Lys	Pro	Leu	Pro	Pro	Glu					
	CAA	ATT	TTG	GAG	AAC	AGG	AAT	GGA	AGC	TTA	AAA	CCT	GGG	AGT	TCT	CAC	CGG	AAG	ACA	AAA	AAG	CCT	CTT	CCC	CCA	ACG	CCT	GAG	GAG	GAC	CAG	ATC	TTG	AAA	AAG	CCA	CTA	CCG	CCT	GAG					
	210	215	220	225	230	235	240	245																																					
748	Pro	Ala	Ala	Ala	Pro	Val	Ser	Thr	Ser	Glu	Leu	Lys	Lys	Val	Val	Ala	Leu	Tyr	Asp	Tyr	Met	Pro	Met	Asn	Ala	Asn	Asp	Leu	Gln	Leu	Arg	Lys	Gly	Asp	Glu	Tyr	Phe	Ile	Leu	Glu					
	CCA	GCA	GCA	GCA	CCA	GTC	TCC	ACA	AGT	GAG	CTG	AAA	AAG	GTT	GTG	GCC	CTT	TAT	GAT	TAC	ATG	CCA	ATG	AAT	GCA	AAT	GAT	CTA	CAG	CTG	CGG	AAG	GGT	GAT	GAA	TAT	TTT	ATC	TTG	GAG					
	250	255	260	265	270	275	280	285																																					
868	Glu	Ser	Asn	Leu	Pro	Trp	Trp	Arg	Ala	Arg	Asp	Lys	Asn	Gly	Gln	Glu	Gly	Tyr	Ile	Pro	Ser	Asn	Tyr	Val	Thr	Glu	Ala	Glu	Asp	Ser	Ile	Glu	Met	Tyr	Glu	Trp	Tyr	Ser	Lys	His					
	GAA	AGC	AAC	TTA	CCA	TGG	TGG	AGA	GCA	GAT	AAA	AAT	GGG	CAG	GAA	GGC	TAC	ATT	CCT	AGT	AAC	TAT	GTG	ACT	GAA	GCA	GAA	GAC	TCC	ATA	GAA	ATG	TAT	GAG	TGG	TAT	TCC	AAA	CAC						
	290	295	300	305	310	315	320	325																																					
988	Met	Thr	Arg	Ser	Gln	Ala	Glu	Gln	Leu	Leu	Lys	Gln	Glu	Gly	Lys	Glu	Gly	Gly	Phe	Ile	Val	Arg	Asp	Ser	Ser	Lys	Ala	Gly	Lys	Tyr	Thr	Val	Ser	Val	Phe	Ala	Lys	Ser	Thr	Gly					
	ATG	ACT	CGG	AGT	CAG	GCT	GAG	CAA	CTG	CTA	AAG	CAA	GAG	GGG	AAA	GAA	GGA	GGT	TTC	ATT	GTG	AGA	GAC	TCC	AGC	AAA	GCT	GGC	AAA	TAT	ACA	GTG	TCT	GTG	TTT	GCT	AAA	TCC	ACA	GGG					
	330	335	340	345	350	355	360	365																																					
1108	Asp	Pro	Gln	Gly	Val	Ile	Arg	His	Tyr	Val	Val	Cys	Ser	Thr	Pro	Gln	Ser	Gln	Tyr	Tyr	Leu	Ala	Glu	Lys	His	Leu	Phe	Ser	Thr	Ile	Pro	Glu	Leu	Ile	Asn	Tyr	His	Gln	His	Asn					
	GAC	CCT	CAA	GGG	GTG	ATA	CGT	CAT	TAT	GTT	GTG	TGT	TCC	ACA	CCT	CAG	AGC	CAG	TAT	TAC	CTG	GCT	GAG	AAG	CAC	CTT	TTC	AGC	ACC	ATC	CCT	GAG	CTC	ATT	AAC	TAC	CAT	CAG	CAC	AAC					
	370	375	380	385	390	395	400	405																																					
1228	Ser	Ala	Gly	Leu	Ile	Ser	Arg	Leu	Lys	Tyr	Pro	Val	Ser	Gln	Gln	Asn	Lys	Asn	Ala	Pro	Ser	Thr	Ala	Gly	Leu	Gly	Tyr	Gly	Ser	Trp	Glu	Ile	Asp	Pro	Lys	Asp	Leu	Thr	Phe	Leu					
	TCT	GCA	GGA	CTC	ATA	TCC	AGG	CTC	AAA	TAT	CCA	GTG	TCT	CAA	CAA	AAC	AAG	AAT	GCA	CCT	TCC	ACT	GCA	GGC	CTG	GGA	TAC	GGA	TCA	TGG	GAA	ATT	GAT	CCA	AAG	GAC	CTG	ACC	TTC	TTG					
	410	415	420	425	430	435	440	445																																					
1348	Lys	Glu	Leu	Gly	Thr	Gly	Gln	Phe	Gly	Val	Val	Lys	Tyr	Gly	Lys	Trp	Arg	Gly	Gln	Tyr	Asp	Val	Ala	Ile	Lys	Met	Ile	Lys	Glu	Gly	Ser	Met	Ser	Glu	Asp	Glu	Phe	Ile	Glu	Glu					
	AAG	GAG	CTG	GGG	ACT	GGA	CAA	TTT	GGG	GTA	GTG	AAG	TAT	GGG	AAA	TGG	AGA	GGC	CAG	TAC	GAC	GTG	GCC	ATC	AAG	ATG	ATC	AAA	GAA	GGC	TCC	ATG	TCT	GAA	GAT	GAA	TTC	ATT	GAA	GAA					
	450	455	460	465	470	475	480	485																																					
1468	Ala	Lys	Val	Met	Met	Asn	Leu	Ser	His	Glu	Lys	Leu	Val	Gln	Leu	Tyr	Gly	Val	Cys	Thr	Lys	Gln	Arg	Pro	Ile	Phe	Ile	Ile	Thr	Glu	Tyr	Met	Ala	Asn	Gly	Cys	Leu	Leu	Asn	Tyr					
	GCC	AAA	GTC	ATG	ATG	AAT	CTT	TCC	CAT	GAG	AAG	CTG	GTG	CAG	TTG	TAT	GGC	GTG	TGC	ACC	AAG	CAG	CGC	CCC	ATC	TTC	ATC	ATC	ACT	GAG	TAC	ATG	GCC	AAT	GGC	TGC	CTC	CTG	AAC	TAC					
	490	495	500	505	510	515	520	525																																					
1588	Leu	Arg	Glu	Met	Arg	His	Arg	Phe	Gln	Thr	Gln	Gln	Leu	Leu	Glu	Met	Cys	Lys	Asp	Val	Cys	Glu	Ala	Met	Glu	Tyr	Leu	Glu	Ser	Lys	Gln	Phe	Leu	His	Arg	Asp	Leu	Ala	Ala	Arg					
	CTG	AGG	GAG	ATG	CGC	CAC	CGC	TTC	CAG	ACT	CAG	CAG	CTG	CTA	GAG	ATG	TGC	AAG	GAT	GTG	TGT	GAA	GCC	ATG	GAA	TAC	CTG	GAG	TCA	AAG	CAG	TTC	CTT	CAC	GCA	GAC	CTG	GCA	GCT	GCA					
	530	535	540	545	550	555	560	565																																					
1708	Asn	Cys	Leu	Val	Asn	Asp	Gln	Gly	Val	Val	Lys	Val	Ser	Asp	Phe	Gly	Leu	Ser	Arg	Tyr	Val	Leu	Asp	Asp	Glu	Tyr	Thr	Ser	Ser	Val	Gly	Ser	Lys	Phe	Pro	Val	Arg	Trp	Ser	Pro					
	AAC	TGT	TTG	GTA	AAC	GAT	CAA	GGA	GTT	GTT	AAA	GTA	TCT	GAT	TTC	GGC	CTG	TCC	AGG	TAT	GTG	CTG	TCC	AGG	TAT	GTG	CTG	GAT	GAT	GAA	TAC	ACA	AGC	TCA	GTA	GGC	TCC	AAA	TTT	CCA	GTC	CGG	TGG	TCC	CCA
	570	575	580	585	590	595	600	605																																					
1828	Pro	Glu	Val	Leu	Met	Tyr	Ser	Lys	Phe	Ser	Ser	Lys	Ser	Asp	Ile	Trp	Ala	Phe	Gly	Val	Leu	Met	Trp	Glu	Ile	Tyr	Ser	Leu	Gly	Lys	Met	Pro	Tyr	Glu	Arg	Phe	Thr	Asn	Ser	Glu					
	CCG	GAA	GTC	CTG	ATG	TAT	AGC	AAG	TTC	AGC	AGC	AAA	TCT	GAC	ATT	TGG	GCT	TTT	GGG	GTT	TTG	ATG	TGG	GAA	ATT	TAC	TCC	CTG	GGG	AAG	ATG	CCA	TAT	GAG	AGA	TTT	ACT	AAC	AGT	GAG					
	610	615	620	625	630	635	640	645																									</												

<i>b</i>		
atk	MAAVILESIFLKRSSQKKKTSPLNFKRLFLTLVHKLSYYEYDFERGRGSKSGSIDVEKITCVETVVPKPNPPPERQIPRRGESSEME	90
atk	QISIIERFPYPFOVYDEGPLYV.FSPTEELRKRWIHOLKNVIRYNSDLVQKYHPCFWIDGOYLCCSQTAKNAMGCCOILENRNGSLKPGSS.	180
tec	MMVSF..PVKINFHSSPQSRDRWVKLLKEEIKNNNNIMIKYHPKFWADGSYOCCROTEKLAPGCEKY.NLFESSI.....	72
src	MGSNKSQPKDAS.QRRRS.L.EPAENVHAGGGAFFASQTP.SKPASADGH. RGPSAAFAP.	56
yes	MGCISKEN.K..SPAIKYRP.ENTP.EPVSTSVSHYGAETTVSPCPSSSA.KGTAVNFSSSL	57
atk	HRKTKKPLPPTREEDQILKKPLPPEPAAAPVS.TSELKVVALYDYMPMNANDLQLRKGDEYF.ILEESNLP.WWRARDK.NGOEGYIPSNY	268
tec	.RKT...LPPAPE...I.KKRRPPPI.PPEENTEE.IVVAMYDFQATEAHDRLRLERGQEI.ILEKNDLH.WWRARDK.....	140
src	AAAEPLFGFNSSDVTSPQAGP..LAGGVTFVALYDYESRTETDLSFKKG.ERLQIVN.NTEGDWWLAHSLSTGOTGYIPSNY	139
yes	SM.TPFGGSSGVTPFGGASSFSVVPSSYPAGLTGGVTFVALYDYEARTEEDLSFKKG.ERLQIVN.NTEGDWWLAHSLSTGOTGYIPSNY	146
SH3-like domain		
atk	VTEAEDSIEMYEYVSKHMTSRQAEQLLKQEGKE.GGFIVRDS.SKAGKYTVSVFAKSTGDPQ.GVIRHVVV.CS.TPOSQVYLAEKHLF.ST	354
tec	YGVYCRNTNRSKAEQLRTEDKE.GGFMVRDS.SQPLTYTVSLYTKFGGEGSSGF.RHYHIKETATSPKKYLAEKHAFGS.	218
src	VAPS.DSIQAEWYFPGKITRRESERLLNAENPRGTEFLVRESEETKGAYCLSV.SDFDNAKGLNVK.HYKIR..KLDSGGFYITSTQFNLSL	226
yes	VAPA.DSIQAEWYFPGKMGRKDAERLLNPNQNGIFLVRESEETKGAYSLSI.RDWDEIRGDNVK.HYKIR..KLDNGGYITTRAQFDTL	233
SH2-like domain		
atk	IPELINYHQ.HNSAGLISRLKYPVSQONKNAPSTAGLGYSWEIDPKD.LTFLKELGTQGFVVKYKQWGRQYDVAIKMIKEGMS.EDEFI	443
tec	IPBIEYHK.HNAAGLVTRLRYPVSTKCKNAETTAGFSYDKWEINPSE.LTFMRELGSGLFGVRLGKWRAQYKVAIKATREGAMCEED.FI	307
src	QQLVAYYSKH.ADGLCHRLTT.VCPTSK..POTQGLAKDAWEI.PRESLRLEVKLQGGCFGEVWMTWNGTTTVAIKTLKPGTMSPEA.FL	311
yes	.QKLVKHYTEH.ADGLCHKELTT.VCPTVK..POTQGLAKDAWEI.PRESLRLEVKLQGGCFGEVWMTWNGTTTVAIKTLKPGTMSPEA.FL	318
ATP-binding site		
atk	EEAKVMMNL.SHEKLVQLYGVCTKQRPFIITEYMANGCLLNLYR.EMRHRFOTOQLLEMCKDVCEAMEYLESKOFLHRDL.AARNCLVNDQ	533
tec	EEAKVMMKLTHPKLVQLYGVCTQOKPIYIVTEFMERGCCLNLFRLRQGH.FSRDMLSMCQDVCEGMEYLERNSFIHRDL.AARNCLVNEAG	397
src	QEAQVMKKLRHEKLVQLYAVVS.EEPIYIVTEYMSKGSLLDFLKGETGKYLRLPOLVDMAAQIASGMAYVERMNYVHRDLRAA.NILVGENL	401
yes	QEAQIMKKLRHDKLVPLYAVVS.EEPIYIVTEFMSKGSLLDFLKEDGKYLKLPOLVDMAAQIADGMAYIERMNYIHRDLRAA.NILVGENL	408
atk	VVKVSDFGLSRYVLDDEYTSVSGSKFPVRWSPPEVLMYSKFPSSKSDIWAFGVLMWEIYSLGKMPYERFTNSETAKHIAQGLRLYRPHLASEK	625
tec	VVKVSDFGMARYVLDQYTSVSSGAKFPVKWCPEVFNYSRFSKSDVWSFGVLMWEIFTEGRMPFEKNTNYEVVMTVTRGHRLLHRPKLAT.K	488
src	VCKVADFGGLARLIEDNEYTARQAKFPIKWTAPAAALYGRFTIKSDVWSFGILLTELTTKGRVPYPGMVNVREVLDOVERGYRMPCPPECPE	493
yes	VCKIADFGGLARLIEDNEYTARQAKFPIKWTAPAAALYGRFTIKSDVWSFGILQTELVTKGRVPYPGMVNVREVLDOVERGYRMPCPQGCPE	500
Substrate-specific domain		
atk	V.YTIMYS.CWHEKA.DERPTFKILLSNIL.DVMDE.ES	659
tec	YLFEVMLR.CWQERPEG.RPSFEDLLRTI..DELVECEETFR	527
src	LHDL.MCQ.CW.RKEPEERPTFEYLQAF.LEDYFTSTEPQYQPGEDL	536
yes	LHEL.MNL.CW.KKDPDERPTFEYIQSF.LEDYFTATEPQYQPGENL	543

FIG. 4 Expression studies of the *atk* transcript. *a*, Expression of the *atk* transcript in a variety of human tissues. The cDNA 14-6 was used to detect a 2.6-kb message which was most abundant in poly(A)⁺ RNA isolated from lung and pancreas. No conclusions could be drawn from the expression of *atk* in placenta due to the absence of signal in this lane using β -actin as a control. RNA size markers are in kilobases. *b*, Expression of *atk* in the lymphoid lineages. A 2.6-kb transcript was detected by cDNA 6G11 in primary tumour cells of patients with chronic lymphocytic leukaemia (B-CLL1 and B-CLL2) and in the B cell line NAD (ref. 61). There was no detectable message in T cells from patients with chronic lymphocytic leukaemia (T-CLL) or in the Jurkat T cell line. The 28S and 18S ribosomal RNAs were used as size markers.



METHODS. A multiple tissue northern blot of poly(A)⁺ RNAs was purchased from Clontech Laboratories for the analysis in *a*. Total RNA was

isolated from the lymphoid lineages using the guanidinium thiocyanate method⁶².

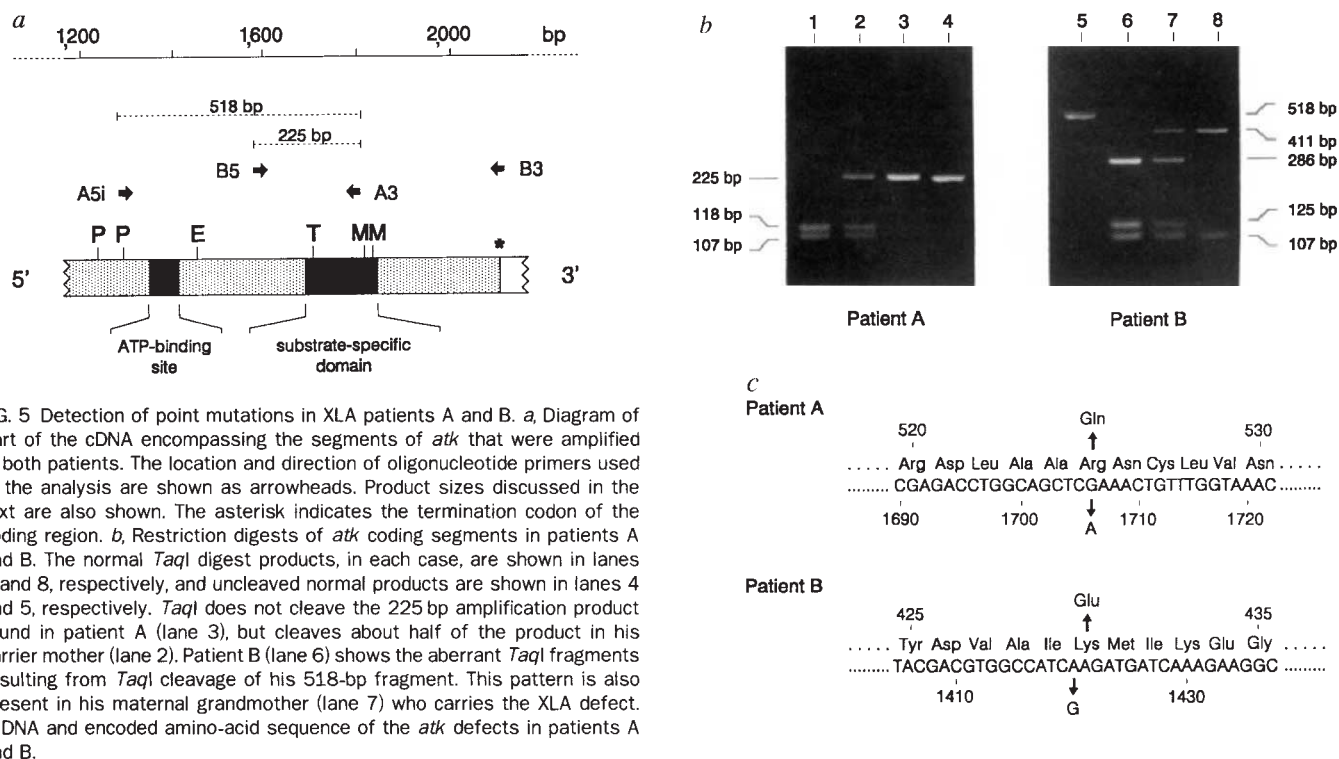


FIG. 5 Detection of point mutations in XLA patients A and B. **a**, Diagram of part of the cDNA encompassing the segments of *atk* that were amplified in both patients. The location and direction of oligonucleotide primers used in the analysis are shown as arrowheads. Product sizes discussed in the text are also shown. The asterisk indicates the termination codon of the coding region. **b**, Restriction digests of *atk* coding segments in patients A and B. The normal *TaqI* digest products, in each case, are shown in lanes 1 and 8, respectively, and uncleaved normal products are shown in lanes 4 and 5, respectively. *TaqI* does not cleave the 225 bp amplification product found in patient A (lane 3), but cleaves about half of the product in his carrier mother (lane 2). Patient B (lane 6) shows the aberrant *TaqI* fragments resulting from *TaqI* cleavage of his 518-bp fragment. This pattern is also present in his maternal grandmother (lane 7) who carries the XLA defect. **c**, DNA and encoded amino-acid sequence of the *atk* defects in patients A and B.

METHODS. RT-based PCR was done as previously described¹⁶. Primer B3 was used for reverse transcription, and then used in combination with either B5 or A5i in the primary PCR region. A second round of PCR was then used to generate the PCR products shown in Fig. 5a and discussed in the text. Primer sequences were A5i:5'-GCAGGCCTGGGATACGGATC-3', A3:5'-

GGAAATTTGGAGCCTACTGAG-3', B5:5'-CCTGAGGGAGATGCGCCAC-3', B3:5'-ATTGGCGAGCTCAGGATTCTTC-3'. Direct DNA sequencing⁴⁶ was done using primers A3 and B5 for patient A, and primers A5i and A3i:5'-CAGACGCCATACAACATGCAC-3' for patient B. Point mutations were confirmed on both DNA strands.

be expressed in the B-cell, but not the T-cell, lineage. Therefore, the failure of pre-B cells in the bone marrow of XLA individuals to develop into mature, circulating B cells could be the result of this gene product failing to fulfill its roles in B-cell signalling. In heterozygous females, pre-B cells whose active X chromosome expresses the mutant gene would not terminally differentiate and proliferate. This latter point may explain why XLA carrier females show completely skewed X-chromosome activity in their B-cell lineage^{4,5}. Finally, we have also shown that two XLA individuals have missense point mutations resulting in amino-acid substitutions at important residues in the putative protein-tyrosine kinase domain. These two mutations are highly suggestive that the normal protein product of *atk* is functioning as a protein-tyrosine kinase. Therefore, naming this gene *atk* aptly describes its predicted protein product and involvement in XLA.

Although the number of genes that encode intracellular protein tyrosine-kinases in the human genome may be quite large⁴⁸, relatively few have been isolated, and the protein products of even fewer have been analysed. But what is known about these genes, based mainly on biochemical evidence, is that their catalytic protein products are likely to play critical roles in the transducing pathways that stimulate cells to grow and adopt specific phenotypes of functional importance. To our knowledge, *atk* is the first member of the human *src* family of oncogenes, which encode intracellular protein tyrosine kinases, to be directly implicated in causing a human genetic disease. Therefore, the study of *atk*, in the context of its normal function and in the XLA phenotype, will provide important new information about the roles *src* protein tyrosine kinases play in cellular signalling. Analysis of a wide range of naturally occurring *atk* mutations in XLA patients will reveal additional amino-acid residues that are important in protein-tyrosine kinases and form the basis for future experimental studies. In this context, note that three members of the *src* family of tyrosine kinases, *src*,

fyn and *lck*, have been inactivated by homologous recombination in mice^{49,50,51}, the *lck* mutation resulting in a thymocyte differentiation defect. Analysis of the *atk* protein product will shed light on the role it plays in B-cell pathways, particularly in view of the fact that *src*-related protein-tyrosine kinases such as *blk*, *fyn*, *lyn* and *lck*⁵²⁻⁵⁵, and others have already been implicated in B-cell activation. Furthermore, studies of the features of *atk* which resemble those of transforming *src* genes may aid in the understanding of oncogene regulation and the events leading to cell transformation. Indeed, it will be interesting to determine whether *atk* is involved in any human neoplastic diseases that are specific to the B-cell lineage. For these reasons, it will be necessary to have a much clearer understanding of the function of *atk*, and *src* genes in general, before somatic gene therapy could be proposed as a means of correcting the XLA defect in pre-B cells of the bone marrow. □

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LETTERS TO NATURE

Quasi-periodic oscillations in X-ray emission from the Seyfert galaxy NGC5548

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EFFORTS to use the variability of X-ray emission from active galactic nuclei (AGNs) as a diagnostic of physical processes in the unresolvable cores have been hampered by the discovery^{1,2} that the variable emission has a 'red noise' character, which seems to indicate the absence of any preferred timescale. The only exception is the claim of a precise periodicity in emission from NGC6814³. Here we analyse archival Exosat observations of the bright Seyfert galaxy NGC5548, and find in the power spectrum a broad peak corresponding to quasi-periodic oscillations (QPOs) with period of ~500 s. Both the frequency and magnitude of the QPOs vary systematically with total X-ray luminosity. Similar behaviour has been seen in compact galactic X-ray binaries, and we suggest that intensity-correlated QPOs may be a generic feature of accretion onto a compact object. If the QPOs in NGC5548 derive from instability or variability in an accretion disc around a black hole, the black hole mass can be only a few hundred thousand solar masses, an uncomfortably small number for most AGN models.

NGC5548 was observed 12 times^{4–6} with the low-energy imaging system⁷ (CMA) and the medium energy⁸ (ME) proportional counter array on board the Exosat observatory. We extracted light-curves from archival data using the Exosat data-base system on the Leicester University Starlink node, and used our own techniques⁹ to estimate the power spectrum and fit models. (Roughly, this involves averaging the usual periodogram in log-log space, giving unbiased independent estimates of known variance, and with distributions close to gaussian.) We use only the ME observations as the CMA count rate is typically an order of magnitude lower. Four observations were excluded because of low signal-to-noise ratio or background subtraction problems. The remainder (see Table 1) were divided into four groups depending on their quality: (1) two long observations

(≥60,000 s) with f.r.m.s. ≥8%, where f.r.m.s. is the fractional root mean square amplitude of variability seen; (2) a long observation with low f.r.m.s. ≤5%; (3) three short observations (≤35,000 s) with high f.r.m.s.; (4) two short observations with low f.r.m.s.

In the best observations, group 1, the power spectra of both observations show three features (Fig. 1c, e): a red-noise component which rises towards low frequencies, a broad peak with a centroid frequency around 2 mHz and a flat component at high frequency due to Poisson noise. The broad feature, which we can loosely refer to as a quasi-periodic oscillation (QPO) cannot be seen directly in the light curve, because many cycles need to be averaged to overcome Poisson noise. In group 2 the red noise is weak, and there is marginal evidence for a similar broad feature (Fig. 1b). In group 3 the individual observations are too short to see much, but we have constructed an ensemble average power spectrum (Fig. 1a) which shows no definite evidence for red noise, but strong evidence for a broad feature. Finally, in group 4, we have again constructed an ensemble average, but there are no significant features.

In the three long observations, models with only a power-law component give values of χ^2 with a probability at or below 10% in two out of three cases. Grouping all three observations, we have $\chi^2 = 67.3$ with 50 degrees of freedom. The probability that a power law can explain all three observations is therefore less than 5%. We can stir in the other two data sets, taking Poisson noise only as the null hypothesis; again, the probability that power-law and/or Poisson noise explains all the data sets is less than 5%. A grouped *F*-test shows that addition of the QPO component is a significant improvement at better than 95% confidence. Of course, χ^2 is a global statistic, and is not very sensitive to local features. On the other hand, our log-power estimates have only approximately a gaussian distribution, and this might lead to incorrect calculation of the likelihood of local peaks. We therefore did a more rigorous test by calculating a periodogram normalized to the values expected from the null hypothesis of power-law/Poisson noise only, and binning up. This gives variables of exactly known distribution, so that we can calculate the significance of any given peak under the null hypothesis. The result is shown in Fig. 2; in three out of five cases a high peak is present at over 95% confidence. The probability of this occurring is only 0.1%. Statistically, the

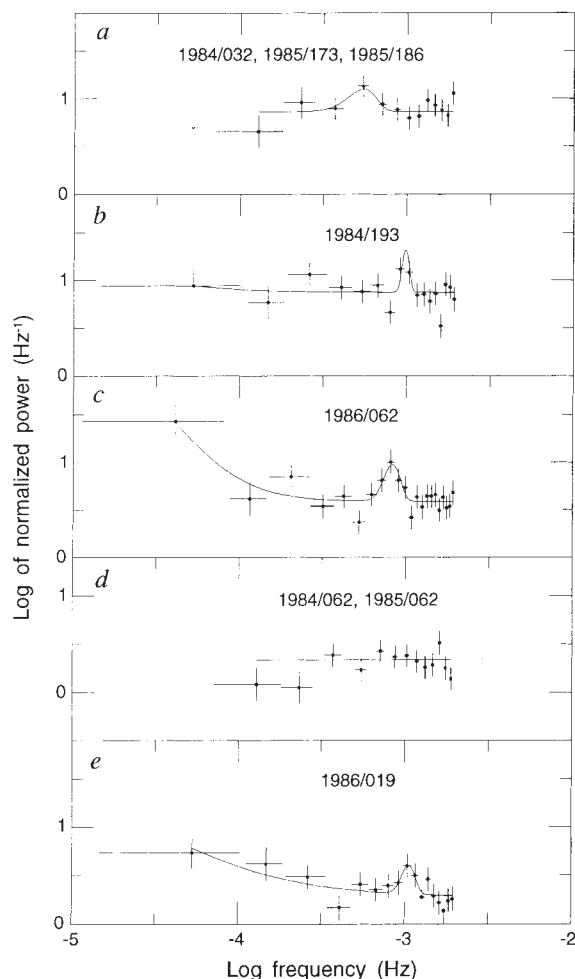
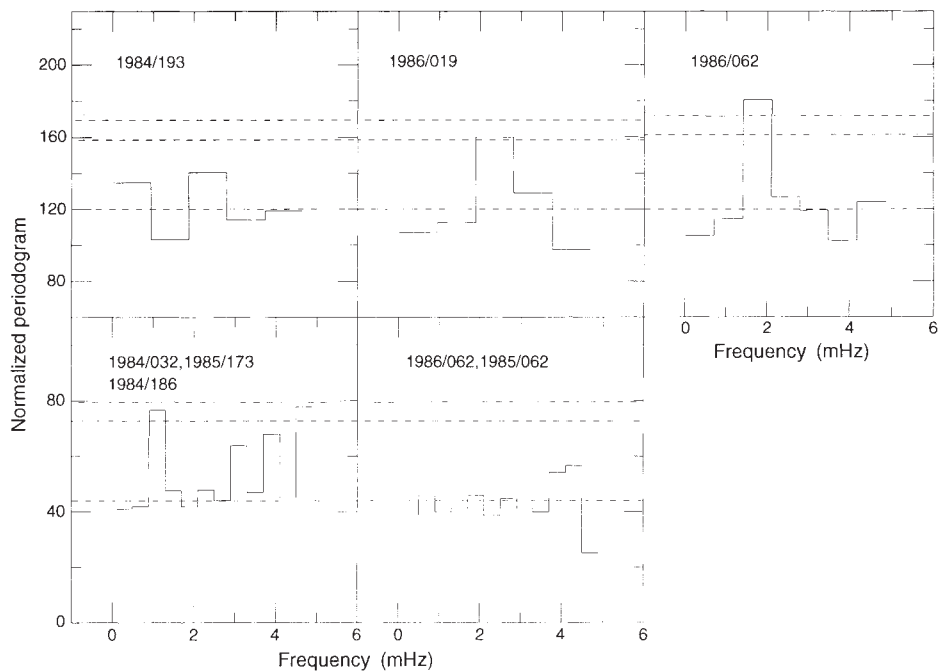


FIG. 1 Power spectra of various observations of NGC5548 arranged in order of source count-rate, together with model fits (see text and Table 1). *a*, Ensemble average spectrum of the three group 3 observations, 1984/032, 1985/173 and 1985/186. The average count rate of the three observations ($3.06 \text{ counts s}^{-1}$) has been taken to represent the group. *b*, Spectrum of the group 2 long observation 1984/193 ($3.12 \text{ counts s}^{-1}$). *c*, Spectrum of the group 1 long observation 1986/062 ($4.17 \text{ counts s}^{-1}$). *d*, Ensemble average spectrum of the two group 4 observations, 1984/062, and 1985/062 (average $4.23 \text{ counts s}^{-1}$). *e*, Spectrum of the group 1 long observation 1986/019 ($5.27 \text{ counts s}^{-1}$). The power spectra and their errors have been estimated using the technique of ref. 9, which involves grouping logarithms of periodogram values. Thus results in estimates that are independent, unbiased for broad features, with exactly known variance and probability distribution close to gaussian after only modest grouping. In all cases, the original light curve started with 100-s binning, and was first normalized to its mean value, so that the resulting power spectrum estimates represent contribution per Hertz to fractional variance. For the long observations (*b, c, e*) the power spectrum was estimated exactly as above, with periodogram estimates grouped 20 at a time, except for the lowest two points, which represent groups of 10. (This is a compromise between having errors as close as possible to gaussian and maximizing the low-frequency 'handle' on the red noise.) For the shorter observations, we preferred to match the frequency resolution in the QPO region rather than maximize information at low frequencies; we therefore split each individual observation into lengths of 5,000 s, which gave 11 independent data sets for each group. A log-periodogram was calculated for each data set, and binned in groups of 2; the ensemble average has 22 independent periodogram points per power spectrum estimate.

FIG. 2 Tests of the significance of the broad QPO feature in each of the data sets of Fig. 1. For the long observations (1984/193, 1986/019 and 1986/062) the null hypothesis was that only red noise was present (other than the experimental Poisson noise). We therefore divided the periodogram values by the best-fit power law (see Table 1), and binned the results so that the QPO features would on average appear mostly in a single high-valued bin. (Note that we chose the bin size, but did not choose the bin placing.) The resulting variables will be χ^2 distributed with $2M$ degrees of freedom, where M is the bin size. (We used $M=60$ for all the long observations.) The dotted lines show the expected mean level of the χ^2 variables, and the levels at which there is a 5% and 1% probability of being exceeded by one or more bins somewhere within the whole periodogram. The QPO is significant at better than 99% confidence for 1986/062, and at $\sim 96\%$ confidence for 1986/109, but only at $\sim 60\%$ confidence for 1984/193. For the short observations, the null hypothesis is that only Poisson noise is present. We worked from the 11 short sections in each case, as in the analysis of Fig. 1. Each periodogram was divided by the Poisson variance and binned in groups of 2; then an ensemble average of the 11 normalized periodograms was taken. This gives χ^2 variables with



44 degrees of freedom. The dotted lines show expected mean, and 95% and 99% confidence, as before. The QPO is significant at $\sim 98\%$ confidence in the data set with the 1984/32, 1985/173 and 1985/186 observations. In the other data set, there are no significant features.

TABLE 1 Summary of observations and model fits

Group	Date (yr/day)	Length (ks)	Intensity (counts s ⁻¹)	f.r.m.s. (%)	Model	A (Hz ⁻¹)	α	B (Hz ⁻¹)	ν_{QPO} (10 ⁻⁴ Hz)	σ (10 ⁻⁴ Hz)	f.r.m.s.-QPO (%)	χ^2 -prob (%)
G1	1986/019	63.8	5.27	7.8	1	2.87 ^{+2.62} _{-1.67}	0.70 ^{+0.56} _{-0.35}					43.0
					2	3.0 ^{+0.43} _{-0.43}	0.90 ^{+0.1} _{-0.06}	1.95 ^{+0.22} _{-0.22}	24 ^{+0.8} _{-0.5}	2.4 ^{+0.2} _{-0.2}	2.42 ^{+0.26} _{-0.24}	78.5
G1	1986/062	85.9	4.17	10.9	1	7.94 ^{+5.56} _{-6.94}	1.27 ^{+25.3} _{-6.94}					6.4
					2	6.33 ^{+0.70} _{-0.63}	1.96 ^{+0.98} _{-0.83}	5.54 ^{+1.11} _{-1.11}	18 ^{+0.2} _{-0.2}	2.1 ^{+1.1} _{-1.1}	3.82 ^{+1.34} _{-1.46}	76.1
G2	1984/193	64.6	3.12	5.3	1	1.35 ^{+4.59} _{-2.93}	0.60 ^{+6.0} _{-0.6}					10.0
					2	0.90 ^{+4.68} _{-0.5}	1.23 ^{+1.54} _{-1.54}	13.41 ^{+1.68} _{-1.68}	22 ^{+3.3} _{-3.4}	1.1 ^{+0.6} _{-0.6}	4.30 ^{+0.38} _{-0.39}	20.2
G3	1984/032	17.6	3.96	10.9	3			5.24 ^{+0.37} _{-0.87}	11 ^{+2.2} _{-1.1}	2.3 ^{+1.0} _{-0.5}	3.89 ^{+1.14} _{-0.75}	79.5
G3	1985/173	18.0	3.22	10.2								
G3	1985/186	24.6	2.0	14.9								
G4	1984/062	33.8	4.65	4.2								
G4	1985/062	27.9	3.81	2.7								

Models: 1, red-noise power law only; 2, red-noise power law plus gaussian; 3, gaussian only.

QPO feature is certainly real, but is probably not present in all observations.

Now we consider possible systematic problems. The background light curves corresponding to Fig. 1a and b were clean; the power spectra showed no peaks. The background light curves corresponding to Fig. 1c and e were less clean, but the power spectra show no significant feature near the observed source QPO, and there is insufficient background variation to account for the QPO signal. The only other known bright source in the field of view of NGC5548 is the BL-Lac object 1E1415.6+2557. However, in an observation immediately after the 1986/062 observation of NGC5548, this object showed no variability¹⁰. We are therefore convinced that the broad peaks in the power spectrum of NGC5548 are related to a physical mechanism that operates in the Seyfert nucleus.

Figure 1 shows the power spectra stacked in order of mean count rate for the observation. The broad feature shifts in frequency. To assess this objectively, we fitted the power spectra to the following function

$$\log [P(\nu)] = \log \left\{ A(10^4 \nu)^{-\alpha} + B \exp \left[-\frac{1}{2} \left(\frac{\nu - \nu_{\text{QPO}}}{\sigma} \right)^2 \right] + C \right\} \quad (1)$$

where $P(\nu)$ is the power spectral density at frequency ν . The first term is a power law to describe the red noise. The second term is a gaussian to describe the broad peak. (C is the constant Poisson noise level.) The best-fit values for parameters are given in Table 1; the uncertainties are 68% confidence, if all the other parameters are allowed to vary. We also show the overall f.r.m.s. amplitude of the QPO feature, obtained by integrating the fitted gaussian over all frequencies. The full function was applied to the data sets of groups 1 and 2. (Table 1 also shows the results of fitting a red-noise component only.) For group 3, there is no significant red noise, so we fitted the QPO part only. Finally, in group 4, we have not attempted to fit any model.

The parameter values of the QPO feature are much better constrained than the red-noise part. Figure 3a and b shows the centroid frequency and f.r.m.s. amplitude of the QPO feature as a function of source intensity. The fitted values for the 1984/193 observation (Figs 1b, 2c) should be treated with caution, as the evidence for the presence of a QPO feature is only marginally ($\sim 85\%$) significant. By eye, it looks as though the formal fit gives a feature which is rather too narrow, and thus at too high a frequency. For completeness, the relevant data are included in Fig. 3 as bracketed points. The result is that the centroid frequency of the QPO increases (roughly linearly) with the source intensity, whereas the strength (f.r.m.s.) of the QPO

decreases as the source brightens. There is no evidence for any change in the fitted width of the QPO.

Within our own Galaxy, both red-noise and broad QPOs are well known in low mass X-ray binaries (LMXBs), and frequently show an identical correlation of frequency and amplitude with source intensity¹¹. The kind of correlations we have seen are those shown by the 'Z sources' when on the horizontal branch of the X-ray colour-intensity diagram. These are objects for which the compact object is normally thought to be a neutron star; the most popular model for the QPOs involves interaction between an accretion flow and the magnetosphere of the neutron star. AGNs, on the other hand, are generally believed to contain massive black holes. Of the three known strong cases for X-ray binaries containing black holes (Cyg X-1, LMC X-3 and A0620-00), the first two show no QPO¹², and for the third there is no information available. Another object often suspected to be a

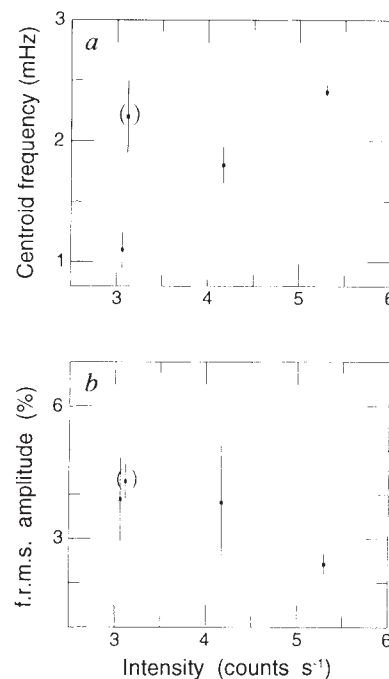


FIG. 3 Dependence of QPO properties on source intensity. a, Centroid frequency of the QPO. b, Fractional r.m.s. amplitude of the QPO. In all cases, the values are the best fit values from model fits, as in Table 1. The values from the 1984/193 observation have been bracketed, as it is not clear whether the QPO feature is real in this case, and the fit does not look realistic.

black-hole candidate on spectral grounds, GX 339-4, does show a 6-Hz QPO¹³, but there is no dynamical proof that the compact object is too massive to be a neutron star. Furthermore the companion star is of low mass¹⁴, whereas both Cyg X-1 and LMC X-3 are high-mass X-ray binaries. It is thought that high-mass X-ray binaries accrete primarily from a stellar wind escaping from the companion star whereas LMXBs accrete by means of Roche-lobe overflow into a disc; a viable hypothesis is that QPOs are a generic feature of disc accretion, regardless of the precise nature of the compact object.

An obvious possibility is that the QPO timescale (~ 500 s) is an orbital period; the modulation could arise directly from the presence of bright spots on an accretion disc^{15,16}, or could drive physical variations, as in the accretion disc corona model¹⁷. For a keplerian orbital period of 500 s, however, if the X-ray emission comes from outside 3 Schwarzschild radii, the black-hole mass can be no larger than 10^6 solar masses ($M_\odot$). This is worryingly small; an estimated dynamical mass for NGC5548, from emission line variability, is $\sim 4 \times 10^7 M_\odot$ (ref. 18). Another possibility is that the timescale represents recurrence in a physical limit-cycle, for example unstable flaring in a pair-plasma. Using the formulae of Moskalik and Sikora¹⁹ and assuming that X-ray emission comes from 3 Schwarzschild radii, we find in this case that the black-hole mass can be no larger than $\sim 2 \times 10^5 M_\odot$, again a worryingly small mass. Finally, oscillations could rep-

resent acoustic modes in an unstable accretion disc²⁰, but predictions for this are less certain, depending on the (unknown) viscosity parameter as well as on the accretion rate. Overall it seems likely that the existence, speed and intensity-correlated behaviour of QPOs in AGNs will provide severe constraints on models. $\square$

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Gamma-ray bursts as collimated jets from neutron star/black hole mergers

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THE distribution of more than 150 γ -ray bursts detected by the BATSE experiment is isotropic on the sky but radially non-uniform^{1,2}. This raises the possibility that bursts are cosmological (at $z \lesssim 1$) and therefore very energetic events, releasing $\sim 10^{50}$ erg sr^{-1} on a timescale of seconds. The coalescence of two neutron stars³⁻⁷ or the accretion-induced collapse of a white dwarf⁸ can release up to 10^{53} erg in the form of neutrino-antineutrino pairs, so that the conversion of $< 1\%$ into γ -rays by annihilation⁹ could generate γ -ray bursts, but in all such models an optically thick wind tends to form^{10,11}, preventing the γ -rays from escaping and converting their energy into kinetic energy of the ejected material. We present here a possible solution to this difficulty. When a stellar-mass neutron star is disrupted by a black hole, it forms a thick disk which emits $\nu\bar{\nu}$ pairs. These neutrinos expel a wind from the disk, but angular momentum conservation means that a clear funnel forms along the rotation axis. Neutrino annihilation within the matter-free funnel can then create γ -rays which escape to the distant observer.

Neutrinos emitted from a neutrinosphere can transfer a small fraction of their energy to ordinary matter essentially through ν -nucleon interactions and the creation of e^+e^- pairs. The deposited energy drives a wind from which γ -rays will emerge only if the mass loss rate $\dot{M} < 10^{-2}(\dot{E}/c^2)$, where $\dot{E}$ is the rate of energy deposition¹⁰. In that case the wind, which is dominated by photons and e^+e^- pairs, becomes highly relativistic with Lorentz factors over 100, allowing a blueshift of the emitted radiation into the γ -ray range^{3,12}. Calculations taking into

account the details of neutrino-matter interaction near the neutrinosphere^{11,13} show, however, that the mass loss rate is orders of magnitude larger than the limit given above. Typically, one obtains

$$\dot{M} \approx \dot{E} \left(\frac{GM}{r_\nu} \right)^{-1} = \left(\frac{2r_\nu}{r_g} \right) \left(\frac{\dot{E}}{c^2} \right) \geq 10 \left(\frac{\dot{E}}{c^2} \right) \quad (1)$$

where M and r_ν are respectively the mass of the compact object and the radius of its neutrinosphere; $r_g = 2GM/c^2$ is the Schwarzschild radius.

This problem could be avoided if there was efficient $\nu\bar{\nu}$ annihilation outside the neutrinosphere, in a region which is not 'polluted' with baryons. We show here that this might be possible in a model where a neutron star (of mass 1.4 solar masses, $M_\odot$) is tidally disrupted by a black hole of a few solar masses ($5 M_\odot$ in our calculations). Roche-lobe overflow by the neutron star occurs for an orbital separation of $\sim 5r_g$ (ref. 14). The subsequent mass transfer can be either dynamical, with a timescale of a few orbital periods (a few milliseconds) or controlled by the rate of gravitational radiation losses by the system¹⁵. This depends on the amount of angular momentum which is stored into the thick disk formed around the black hole. Nevertheless, as the timescale for the emission of gravitational radiation is also extremely short (a few tens of milliseconds) the difference between the two situations is probably unimportant for their future evolution.

A detailed description of the coalescence process (three-dimensional hydrodynamics, decompression of neutron star material, analysis of disk stability over a period of several seconds and so on) is beyond our present scope. We simply assume that in the resulting disk the neutrinosphere will have its inner limit at 3 Schwarzschild radii and a circular section of radius $2r_g$ (see Fig. 1 for a schematic representation of the system geometry). A wind will be driven by the neutrino emission so that γ -rays cannot be expected directly from the disk. However, the wind material, which has a large angular momentum, will flow away from the system vertical axis, leaving there a baryon-free region which can be reached by neutrinos¹⁶. If efficient $\nu\bar{\nu}$ annihilation can occur along the axis, then a fireball will be created, from which γ -rays will emerge (Fig. 1).

To test this possibility we have made an order-of-magnitude estimate for the energy $q_{\nu\bar{\nu}}(r)$ released by $\nu\bar{\nu}$ annihilation on the vertical axis. We have neglected the aberration and the

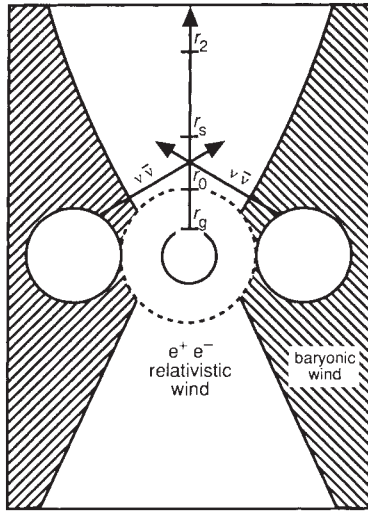


FIG. 1 Schematic representation of the system geometry. The inner and outer radii of the toroidal neutrinosphere lie at 3 and $5 r_g$ respectively. Because of conservation of angular momentum, the material outflow from the neutrinosphere leaves a baryon-free funnel along the vertical axis of the system. Neutrinos annihilating in the funnel produce a relativistic wind which is the source of the γ emission. r_0 , r_s and r_2 are, respectively, the wind starting point, the sonic radius and the distance at which $\Gamma=2$.

Doppler shift of the neutrinos, and the effects of general relativity. The neutrinos then move on straight lines, making the calculation of $q_{\nu\bar{\nu}}$ (ref. 9) simple

$$q_{\nu\bar{\nu}}(r) \approx 3 \times 10^{23} T_\nu^8 \int (1 - \mathbf{\Omega}_\nu \cdot \mathbf{\Omega}_{\bar{\nu}})^2 d\Omega_\nu d\Omega_{\bar{\nu}} \\ = 3 \times 10^{23} T_\nu^8 \Phi(r) \text{ erg cm}^{-3} \text{ s}^{-1} \quad (2)$$

where T_ν is the temperature at the neutrinosphere in MeV and $\mathbf{\Omega}_\nu$ (and $\mathbf{\Omega}_{\bar{\nu}}$) are unit vectors along the direction of propagation of neutrinos interacting at a radius r . The aberration angle and Doppler shift can be estimated from the rotation velocity of the disk material $\beta = v/c \sim \frac{1}{3}$

$$\Delta\alpha \sim \beta \sin \alpha \quad \frac{\Delta\nu}{\nu} = \frac{\sqrt{1-\beta^2}}{1-\beta \cos \alpha} - 1 \quad (3)$$

where α is the angle between the neutrino direction and the disk velocity in the observer's frame. Both the aberration angle and Doppler shift are moderate ($\Delta\alpha \leq 20^\circ$, $\Delta\nu/\nu \approx 0.1$) and should not greatly affect the result of equation (2). The effects of general relativity (gravitational redshift and deflection of the neutrino trajectories) on $q_{\nu\bar{\nu}}$ are probably more important. Deflection increases the efficiency of $\nu\bar{\nu}$ annihilation (by allowing more head-on collisions) whereas gravitational redshift reduces it, the overall effect being difficult to calculate. Also, because of deflection, the total momentum of the annihilating neutrinos will be directed towards the black hole below some radius $r_0 \approx 3r_g$. The flow of photons and e^+e^- pairs moving outwards will therefore start at r_0 while all the energy deposited at $r < r_0$ will ultimately fall into the hole.

The luminosity of a cosmological γ -ray burst is of the order of $(10^{51}/4\pi) \text{ erg s}^{-1} \text{ sr}^{-1}$. To generate such an energy along the vertical axis of the system, the temperature of the neutrinosphere must satisfy the constraint

$$\int_{r_0}^{\infty} q_{\nu\bar{\nu}}(r) r^2 dr = 3 \times 10^{23} T_\nu^8 \int_{r_0}^{\infty} \Phi(r) r^2 dr = \frac{10^{51}}{4\pi} \quad (4)$$

This supposes that the fireball does not expand too much in directions perpendicular to the axis. This can be achieved if the

wind coming from the disk can confine the flow of photons and e^+e^- pairs until it reaches ultra-relativistic velocities (see below). We have calculated the integral of $\phi(r)$ in equation (4) for the adopted system geometry, leading to $T_\nu = 4.76 \text{ MeV}$ and a total neutrino luminosity $L_\nu = \Sigma_\nu \times 3 \times 10^8 \sigma T_\nu^4 = 1.2 \times 10^{54} \text{ erg s}^{-1}$, where $\Sigma_\nu = 40\pi^2 r_g^2$ is the surface of the neutrinosphere. Only 0.1% of the neutrino energy is therefore converted into γ -rays. With such a large value of L_ν , all the energy available from the disruption of the neutron star (several times 10^{53} erg) will be released in less than one second, the duration of the observed burst being $(1+z)$ times longer, where z is the redshift.

We assume that the relativistic flow is confined along the vertical axis within a solid angle $\Delta\Omega$. Following Flammang¹⁷ and Paczynski³, we can write the flow equations as

$$(U + P)\Delta\Omega r^2 v Y^2 = L_{\nu\bar{\nu}} \quad (5)$$

$$TY = T_0 \quad (6)$$

$$\frac{dL_{\nu\bar{\nu}}}{dr} = \Delta\Omega r^2 \left(1 - \frac{r_g}{r}\right)^{1/2} q_{\nu\bar{\nu}} \quad (7)$$

where T_0 is a constant, $Y = (1 - r_g/r)^{1/2} (1 - v^2/c^2)^{-1/2}$ and all other symbols have their usual meaning.

Substituting equation (6) into (5), one finds

$$\Delta\Omega^{1/3} (1 + \eta(T)) \sigma T_0^4 \beta (1 - \beta^2) = \frac{L_{\nu\bar{\nu}}}{r^2} \left(1 - \frac{r_g}{r}\right) = F(r) \quad (8)$$

where $\beta = v/c$ and $\eta(T)$ decreases from 7/4 for ultra-relativistic electrons to 0 after annihilation. An approximate solution of equation (8) can be readily obtained if $\eta(T)$ is taken constant. The sonic radius r_s , where $\beta = \beta_s = 1/\sqrt{3}$, is computed from the condition $(dF/dr)_s = 0$ which gives $r_s/r_g = 5.12$ and $T_0 = 555 (1 + \eta)^{-1/4} \text{ keV}$. The results for β and the Lorentz factor $\Gamma = (1 - \beta^2)^{-1/2}$ have been represented in Fig. 2 as a function of r . At large distances, Γ is proportional to r and T to r^{-1} . We can suppose that the wind coming from the disk will be able to confine the fireball as long as its temperature (and therefore its pressure) remains larger. If this is the case until $\Gamma \geq 2$, relativistic beaming (of opening angle $\tan^{-1}(1/\Gamma) < 30^\circ$) will limit the lateral extension of the flow when direct confinement stops. The Lorentz factor in the fireball reaches $\Gamma = 2$ at a radius $r_2 \approx 150 \text{ km}$ and a temperature $T_2 \approx 200\text{--}300 \text{ keV}$. It is difficult to estimate the wind temperature T_w at r_2 , but in spherical symmetry and with $T_\nu \approx 4\text{--}5 \text{ MeV}$, $T_w \approx 500\text{--}600 \text{ keV}$ at $r = 150 \text{ km}$. The actual wind will be far from spherical symmetry, but confinement of the fireball by the wind does not seem unrealistic.

How does the resulting γ -ray burst compare to the observations? First, one would expect a thermal spectrum at a temperature $T \approx 500/(1+z) \text{ keV}$, in direct conflict with the observed

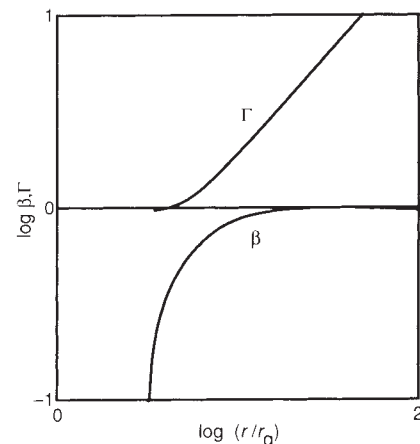


FIG. 2 Values of β and Γ in the relativistic wind. At large distances Γ becomes proportional to r .

power laws^{18,19}. This is a general problem for all models trying to explain γ -ray bursts in terms of an expanding fireball. A possible way out is to invoke various interactions between the fireball and an external medium¹⁶ to produce the nonthermal spectrum, but the problem is far from being settled. Also, the peak fluxes predicted by our model may be rather low ($\sim 10^{-6} h^2 \text{ erg cm}^{-2} \text{ s}^{-1} \text{ keV}^{-1}$ for $z=1$, h being the Hubble constant in units of $100 \text{ km s}^{-1} \text{ Mpc}^{-1}$), especially if $h=0.5$. Gamma-ray bursts show a large variety of time profiles and durations¹⁸. Our model may naturally explain short bursts with smooth profiles. For those bursts showing a complex time structure and lasting up to 100 s, Narayan *et al.*²⁰ have proposed a mechanism involving a series of reconnection events in an ultra-strong magnetic field produced by disk instabilities or differential rotation. We suggest that, at least in some cases, the neutrino annihilation model could also lead to variability at the millisecond scale. If the disk is highly turbulent, induced shocks will cause a succession of short pulses of neutrino emission when they arrive near the neutrinosphere (in the same way that an ultraviolet flash is formed when the shock emerges at the surface in a type II supernova explosion). If these pulses carry enough energy, they can produce (after $\nu\bar{\nu}$ annihilation) the characteristic temporal behaviour of the complex bursts (R.M., manuscript in preparation).

Finally, we consider the burst statistics, assuming that the wind confines the fireball within an angle $\theta \approx 30\text{--}50^\circ$ from the vertical axis of the system. The fraction of detectable bursts is then $f=1-\cos\theta=0.15\text{--}0.40$. The rate $\tau_{\text{BH/NS}}$ of black hole/neutron star mergers has been estimated²¹ to be $10^{-5}\text{--}10^{-6} \text{ yr}^{-1}$ per galaxy and the product $f\tau_{\text{BH/NS}}$ therefore

remains comparable to the rate of γ -ray bursts, $\sim 10^{-6} \text{ yr}^{-1}$ per galaxy²².

After completing this work, we became aware of the work in ref. 23, where the idea of using neutrino-antineutrino annihilation along the system axis is also proposed. $\square$

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Formation of long-lived gas-phase antiprotonic helium atoms and quenching by H_2

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IN 1964 Condo¹ suggested that the decay characteristics of negative (π^- and K^-) mesons in helium bubble chambers could be explained by the capture of these particles in large-angular-momentum metastable orbitals of exotic helium atoms. Russell² predicted that similar ‘atoms’ might be formed by antiprotons in liquid helium. Nearly two decades later the postulated metastability of K^- and π^- mesons in liquid helium was observed experimentally^{3–5}. We recently observed⁶ that about 3% of the antiprotons stopped in liquid helium survive for several microseconds before annihilating in the helium nuclei. This is more than a million times longer than the typical (picosecond) lifetimes of antiprotons that come to rest in matter, and it represents the signature of the formation of metastable antiprotonic atoms. Here we show that the same phenomenon is observed in gas-phase helium, but that surprisingly the lifetime of the ‘atoms’ is the same as in the liquid phase, despite the reduction in collisional de-excitation. In addition, we

show that the presence of trace amounts of hydrogen gas greatly reduces the lifetime, suggesting that a single collision with H_2 is sufficient to destroy the metastability.

A recent theoretical account⁷ deals quantitatively with the metastable antiprotonic atom (Fig. 1). The description given below is semiquantitative, but reveals its important properties. A decelerating antiproton is likely to form an exotic atom only when its kinetic energy falls below the helium ionization energy ($I_0=24.6 \text{ eV}$). The captured antiproton ($\bar{p}$) should go into a state with principal quantum number n_0 near $(M^*/m_e)^{1/2}$ (where M^* is the reduced mass of the antiproton), as this value, 38 in the case of $\bar{p}^4\text{He}$, gives the best overlap with the wave function of the displaced electron and the binding energy is similar (Fig. 1). The energy-level spacing in this region is $\sim 2 \text{ eV}$, much smaller than the electron ionization energy, which is nearly equal to the ionization energy of an ordinary helium atom. For states of large angular momentum ($l>34$), fast Auger transitions liberating the remaining electron are energetically impossible, and de-excitation to lower- n states can only occur either by ‘forbidden’ Auger transitions ($\Delta l>3$) or by a series of slow radiative transitions. The degeneracy of states with different l but the same n is removed by the $\bar{p}$ - e^- interaction, and the coupling with surrounding helium atoms is weak because the Pauli principle prohibits the electron cloud from penetrating neighbouring atoms (unlike protonium, $\bar{p}\text{p}$, which has no electron). Annihilation of the $\bar{p}$ through the Stark effect⁸ is accordingly improbable. We thus expect the $\bar{p}$ to occupy metastable states with high angular momentum. These are analogous to the high-spin rotational states of nuclei known as nuclear yrast bands.

Atomic states at large l -values are well represented by classical orbits. The $\bar{p}$ orbits at a frequency $\sim 1/38$ that of the $1s$ electron, which therefore tends to respond to the location of the $\bar{p}$. This is a typical instance of the Born-Oppenheimer condition for a diatomic molecule (calculations for this metastable atom have been based on this approximation^{9,10}). Consequently, the elec-

tron charge cloud tends to be polarized on the opposite side of the nucleus to the $\bar{p}$. This slows down the radiative process by a further factor of ~ 3 (ref. 7).

Such a metastable $\bar{p}\text{He}^+$ atom possesses some of the features of both atoms and molecules. We could call it an exotic helium atom, as one electron is replaced by a $\bar{p}$; or an exotic hydrogen atom, as $\bar{p}\text{He}^{2+}$ is an extended pseudo-proton; or an exotic diatomic molecule, where one nucleus has a charge of $+2$ and the other 'nucleus' has a charge of -1 . Despite the electrostatic attraction between the two 'nuclei', the electron cloud helps to prevent immediate collapse for the reasons stated above. The formation, structure and reactions of such 'atomcules' are thus of great interest.

The large angular momentum needed for metastability is provided by the impinging antiproton. On average, the antiproton loses energy near the end of its range in steps of ~ 20 – 25 eV by exciting or ionizing helium atoms. The collision energy at the formation stage (after the last but one encounter) will be distributed up to about this value. The maximum angular momentum brought in by the antiproton can then be estimated by multiplying the corresponding momentum (~ 0.2 MeV/ c) by the Bohr radius, giving $\sim 40 \hbar/2\pi$. Above this value, the population of l states should rapidly fall off. Similar considerations⁷ give the delayed annihilation fraction as $\sim 4\%$.

The time spectrum of delayed annihilation, $n(t)$, depends on the initial population of the metastable states and on their decay rates. As a typical metastable level has (according to the model) a lifetime of $\sim 2 \mu\text{s}$ and there is a series of such levels in the

cascade, we expect that the time spectrum will not be a single exponential but will follow a more complex curve. The overall lifetime could thus be much longer than the typical single-transition lifetime. But the observed mean lifetime in liquid helium was only $\sim 3 \mu\text{s}$. One explanation for this difference might be that the initial state is perturbed by collisions in the (high-density) liquid phase. In the first stage of our experiments at the Low-Energy Antiproton Ring (CERN), we concentrated on reproducing the effect in helium gas to see whether the $\bar{p}$ annihilation is subject to a longer delay in the gas than in the liquid phase.

The monoenergetic $\bar{p}$ beam of momentum 100 MeV/ c (kinetic energy 5.6 MeV) traversed a 100- μm plastic scintillator before entering a vessel containing helium gas, where almost all the $\bar{p}$ stopped. Charged mesons resulting from $\bar{p}$ annihilation were detected by scintillation counter arrays almost entirely surrounding the stopping point. The time delay between each antiproton's arrival in the gas and its annihilation was digitized. It was important to minimize the detection inefficiency of the beam scintillator, as undetected antiprotons may follow a given antiproton within the time range of interest, and produce fake delayed events. We only accepted annihilations in which three simultaneous charged particles were recorded in the scintillation counters. This requirement eliminated events of 2.2- μs mean lifetime caused by $\pi^+ \rightarrow \mu^+ \rightarrow e^+$ decay by normal ('prompt') annihilation.

Figure 2 shows time spectra of $\bar{p}$ annihilation in ^4He gas (3 atm), ^3He gas (3 atm) and liquid ^4He . The fraction and spectral shape of the delayed component are almost the same in the gas as in the liquid phase over a gas pressure range of 2–10 atm. This tends to contradict the hypothesis of collisional excitation or de-excitation of the metastable atom in pure He.

The above liquid helium experiment⁶ (done at the National Laboratory for High Energy Physics (KEK) in Japan) established only marginally the expected complex behaviour in the

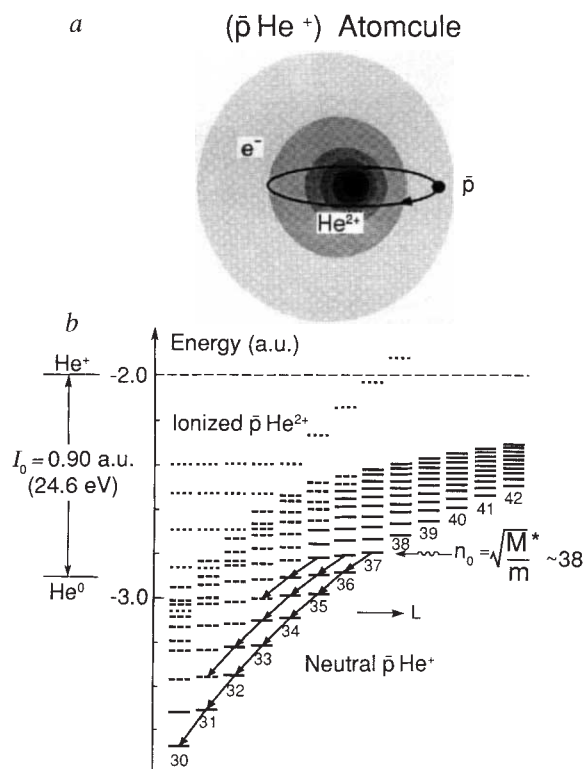


FIG. 1 The metastable $\bar{p}\text{He}^+$ atomcule, based on the theoretical calculations of Ohtsuki⁷. *a*, Antiproton in a large- l orbital ($n=38$, $l=37$) following a 'classical' trajectory around the He^{2+} nucleus with a frequency much lower than that of the electron in the ground state, dominantly $1s$. The solid line shows the $\bar{p}$ trajectory in the rest frame of He^{2+} , viewed at an angle to the plane of its orbit. The electron cloud with density distribution as shown is polarized oppositely with respect to the $\bar{p}$ location. As the $\bar{p}$ moves, the electron cloud circulates accordingly. *b*, The level scheme for the relevant region. The solid lines are for the metastable states, the dashed lines are for short-lived states ($\tau < 2.5$ ns, dominated by Auger transitions), and the dot-dashed lines are for ionized $\bar{p}\text{He}^{2+}$ states. The main stream of slow radiative transitions is indicated by arrows.

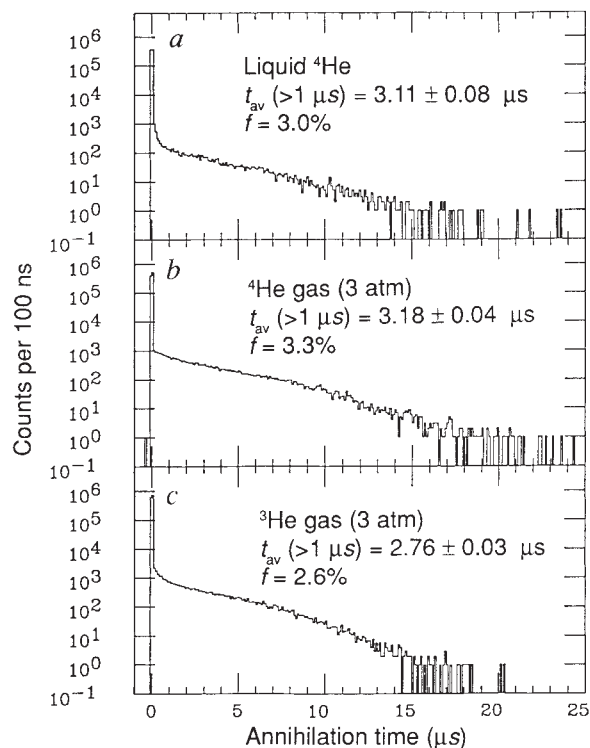


FIG. 2 Comparison of the time spectra of $\bar{p}$ annihilation in ^4He gas (*b*) and ^3He gas (*c*) at 3 atm pressure with the liquid helium data (*a*) taken at KEK⁶. The typical values $t_{av}(>t_0)$ for $t_0=1 \mu\text{s}$ and the delayed fractions f are shown.

time spectrum. Our present experiment has good enough statistics to reveal the presence of this feature for annihilations delayed by more than 5 μs . The spectrum $n(t)$ cannot be represented by a simple analytical expression; instead we define an 'average lifetime' $t_{\text{av}}(>t_0)$ as

$$t_{\text{av}}(>t_0) = \frac{\int_{t_0}^{50\mu\text{s}} t n(t) dt}{\int_{t_0}^{50\mu\text{s}} n(t) dt} - t_0 \quad (1)$$

for an initial time t_0 . The numerical values of t_{av} and the delayed fraction f deduced from the experimental spectra are indicated in Figs 2 and 3.

We have also studied the isotope dependence of the delayed annihilation by comparing the time spectra in ^4He and ^3He gas (Fig. 2). The average annihilation time for $\bar{p}$ in ^3He is $15 \pm 2\%$ shorter than that for $\bar{p}$ in ^4He . A crude theoretical estimate⁷ indicates that the overall cascade time is proportional to $(M^*)^2$. The $\bar{p}$ reduced mass is considerably different in ^4He and ^3He , and we estimate the lifetime ratio to be 1.14, which agrees well with the observed isotope effect. This supports the atomic model.

Although we did not see the difference in behaviour originally expected in going from liquid to gaseous helium, the time spectrum is sensitive to small admixtures of foreign gases. Great care was necessary in preparing the gas target; we baked the gas vessel and purified the helium gas using a molecular sieve. To study the impurity effect we added hydrogen gas at low concentrations. The time spectrum was strongly affected, the part corresponding to the metastable states being quenched to a degree depending on the hydrogen concentration (Fig. 3). At 400 p.p.m. H_2 the lifetime was reduced to $\sim 0.37 \mu\text{s}$. Even at 20 p.p.m. H_2 some effect was seen, whereas no quenching was found when we added up to 5% Ne gas. The Ne gas is a good medium for storing the metastable $\bar{p}\text{H}^+$ atoms, although no metastable atom is formed in pure Ne gas itself.

A question raised by this quenching effect is whether the metastability is destroyed by collisions with impurity atoms. The total delayed fraction remains nearly constant no matter how

short the lifetime becomes. This suggests that the quenching is due to collisions after formation, which, with a time distribution $\exp(-\nu t)$, remove the $\bar{p}$ from a metastable state to another state with lifetime shorter than the mean collision time.

The simplest assumption is that the quenching rate is given by $\nu = \sigma n_i v$, where σ is the quenching cross-section, n_i is the impurity density and v is the relative velocity. The thermal velocities of metastable atoms and hydrogen molecules are $\sim 0.1 \text{ cm } \mu\text{s}^{-1}$ and $\sim 0.16 \text{ cm } \mu\text{s}^{-1}$, respectively, and the impurity density n_i is $3.2 \times 10^{16} \text{ cm}^{-3}$ for 400 p.p.m. H_2 in 3 atm He gas. The observed quenching rate of $2.7 \mu\text{s}^{-1}$ would yield a quenching cross-section of $\sim 5 \times 10^{-16} \text{ cm}^2$, a typical value for a geometrical atomic cross-section. This means that a single collision with a H_2 molecule destroys the metastability nearly completely.

What mechanism can cause such violent destruction? Collisions with impurity atoms or molecules could speed up the deexcitation of the metastable $\bar{p}\text{He}$ atoms by carrying away energy and angular momentum, or could induce chemical reactions. Experiments on the reactions of our atomcules in various gases are in progress. With large impurity concentrations (true gas mixtures) it should be possible to adjust the initial population of l , as enough foreign atoms would be present to change the $\bar{p}$ energy distribution at the formation stage. Adding gas with a lower ionization potential than helium should, for example, result in lower l -values, and reduce both the lifetime and the delayed fraction. We are planning detailed spectroscopic studies of the 'atomcules'. $\square$

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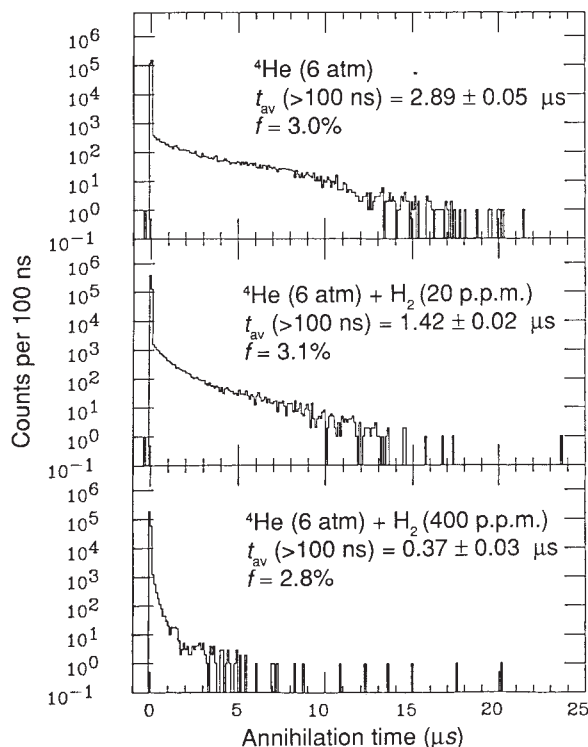


FIG. 3 Quenching of the $\bar{p}\text{He}^+$ metastability in gaseous helium induced by hydrogen gas impurities. The typical values $t_{\text{av}}(>t_0)$ for $t_0=100 \text{ ns}$ and the delayed fractions f are shown.

Controlling chaos in the Belousov–Zhabotinsky reaction

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DETERMINISTIC chaos is characterized by long-term unpredictability arising from an extreme sensitivity to initial conditions. Such behaviour may be undesirable, particularly for processes dependent on temporal regulation. On the other hand, a chaotic system can be viewed as a virtually unlimited reservoir of periodic behaviour which may be accessed when appropriate feedback is applied to one of the system parameters¹. Feedback algorithms have now been successfully applied to stabilize periodic oscillations in chaotic laser², diode³, hydrodynamic⁴ and magnetoelastic⁵ systems, and more recently in myocardial tissue⁶. Here we apply a map-based, proportional-feedback algorithm^{7,8} to stabilize periodic

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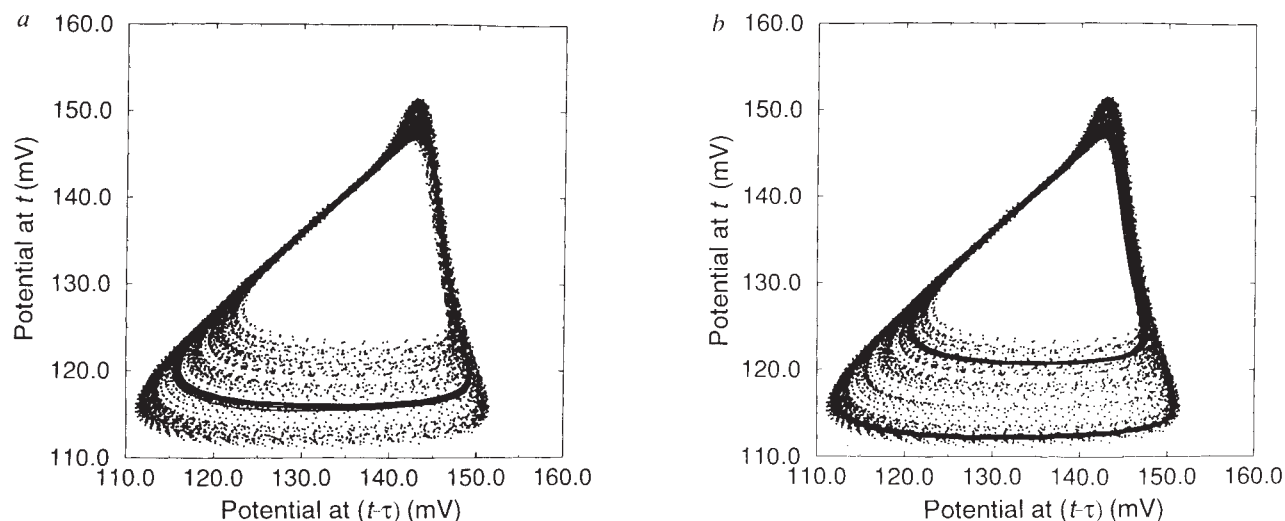


FIG. 1 Stabilized period-1 and period-2 limit cycles embedded in strange attractor of the BZ reaction. Scattered points show chaotic trajectory in time-delay phase space ($\tau=13$ s) with a reactor residence time of 2.8×10^3 s and concentrations, after mixing, of [malonic acid] = 2.22×10^{-1} M, $[\text{Ce}_2(\text{SO}_4)_3] = 4.50 \times 10^{-4}$ M, $[\text{NaBrO}_3] = 1.02 \times 10^{-1}$ M and $[\text{H}_2\text{SO}_4] = 0.200$ M. Solid curves show (a) period-1 limit cycle stabilized by using $A_s = 33.0$ mV and $g = 18.0$ in equation (1) and (b) period-2 limit cycle stabilized by using $A_s = 26.9$ mV and $g = 33.4$. Reaction was carried out in a continuously stirred tank reactor (volume 34.0 ml, stirring rate 1,800 r.p.m.)

maintained at 28.0 ± 0.1 °C and fed with separate solutions of malonic acid, cerous sulphate and sodium bromate. Two peristaltic pumps were used, with the malonic acid solution delivered at a fixed flow rate by one, and the cerium and bromate solutions (acidified with sulphuric acid) delivered by the other at a rate regulated by a computer. The potential of a bromide-ion-selective electrode was monitored and collected at a frequency of 10 Hz by a 16-bit data-acquisition board. Moving averages of 10 values were calculated and stored each second, and these values were numerically filtered using a 5-s characteristic period.

behaviour in the chaotic regime of an oscillatory chemical system: the Belousov-Zhabotinsky reaction.

The dynamical behaviour of a chaotic system can be visualized by the strange attractor (an attractor in which the trajectory never exactly repeats itself) traced out by its trajectory in phase space. An infinite number of unstable periodic orbits are embedded in such an attractor, each characterized by a distinct number of oscillations per period. In low-dimensional systems, these orbits are simple saddle-type limit cycles with an attracting stable manifold and a repelling unstable manifold. A general algorithm for stabilizing such orbits, based on targeting the stable manifold of the limit cycle by applying perturbations to a system constraint, has been developed by Ott, Grebogi and Yorke¹ (OGY). The stable manifold is found in a particular Poincaré section in the phase space, and it is targeted in this section each period of oscillation. For systems exhibiting low-dimensional chaos characterized by effectively one-dimensional maps, the OGY method can be reduced to a simple map-based algorithm^{7,8}. The simplified method is more convenient in experimental applications because it minimizes the targeting procedures, and it has been used to control chaos in laser² and diode³ systems.

The best-studied example of an oscillatory chemical system is the Belousov-Zhabotinsky (BZ) reaction, in which Ce(IV)/Ce(III) catalyses the oxidation and bromination of $\text{CH}_2(\text{COOH})_2$ (malonic acid) by BrO_3^- in H_2SO_4 . If the reaction is carried out in a continuous-flow stirred-tank reactor, the flow rate of the reactants into the tank ultimately determines whether the system exhibits steady-state, periodic or chaotic behaviour. Here we use conditions similar to the low-flow-rate Texas experiments⁹⁻¹¹, which ensure that the system is maintained within the chaotic regime (see Fig. 1). An important difference in our experiment is that we apply feedback to the system by perturbing the rate at which the cerium and bromate solutions are fed into the tank (the flow rate of the malonic acid being fixed), permitting the targeting and stabilization of periodic behaviour within the chaotic regime.

Figure 1a shows the strange attractor and the stabilized period-1 limit cycle for the BZ reaction. The time-delay phase

portrait was reconstructed *in situ* from smoothed values of the potential of a bromide electrode. Except for small positive and negative perturbations to the flow rate of the bromate and cerium reactant stream, the operating conditions were identical for the chaotic and periodic behaviour. Figure 1b shows the stabilized period-2 limit cycle embedded in the strange attractor, again obtained with the same average reactant-stream flow rate. The oscillatory behaviour can be switched between period-1, period-2 and chaos by simple adjustments of the proportional feedback. Each time the controlling experiment was repeated (more than 10 times), a bifurcation diagram was first constructed to locate a suitably wide range of chaotic behaviour arising from a period-doubling cascade. The flow rate of the bromate and cerium reactant stream was chosen as the bifurcation parameter, as this choice gave the widest range of period-doubling chaos.

The control algorithm takes advantage of the predictable evolution of a chaotic system in the vicinity of a fixed point in the next-amplitude map, corresponding to a particular unstable periodic orbit. Shown in Fig. 2a is the next-amplitude map for the strange attractor and the period-1 orbit shown in Fig. 1a. The position of the period-1 fixed point is given by the intersection of the map with the bisectrix, where system state $A_{n+1} = A_n$. As the chaotic system traverses the attractor, the region near the fixed point is eventually visited, and the control algorithm is then activated. Control is achieved by perturbing a system constraint such that the fixed point is targeted on each return.

A shift of the map occurs on varying a system constraint, and this shift can be used to target any particular fixed point. Shown in Fig. 2b is a next-amplitude map constructed from the Györgyi-Field¹² model of the BZ reaction with concentrations and residence time similar to the experimental values for Fig. 2a. The inset shows a blowup of the map around the period-1 fixed point (right) and the shifted map (left) obtained at a slightly different value of the bifurcation parameter μ (the flow rate of the bromate and cerium reactant stream). For small perturbations, the shift in the linear region around the fixed point is directly proportional to the variation in μ . The map can therefore be shifted to target the fixed point by applying a

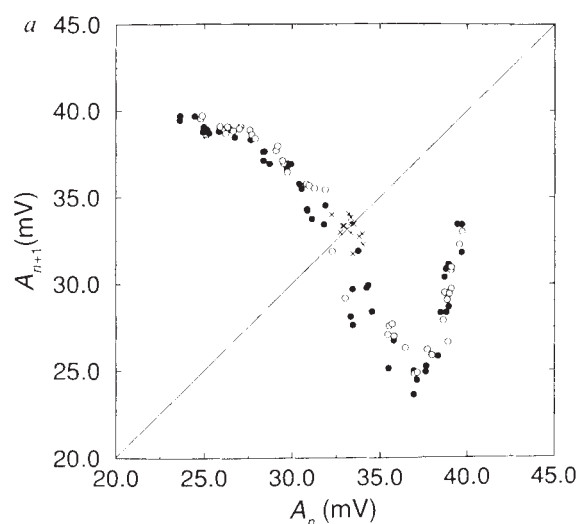
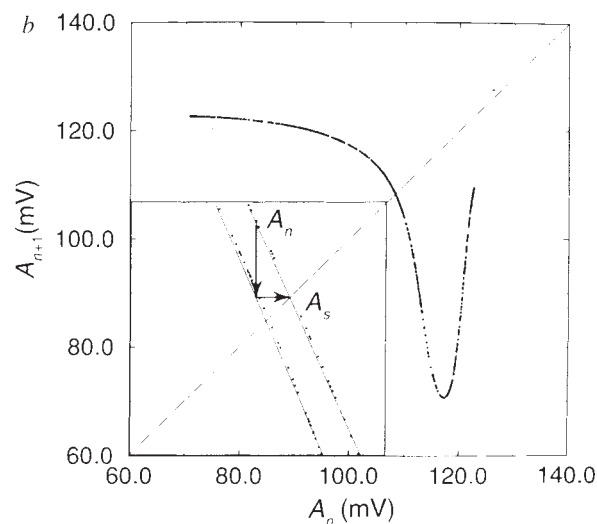


FIG. 2 *a*, Next-amplitude map before (●) and after (○) controlling period-1 and period-2, respectively, as shown in Figs 1 and 3*a*. Also shown are values (×) near fixed point during control corresponding to period-1 limit cycle trajectory in Fig. 1*a*. *b*, Next-amplitude map calculated from three-variable Györgyi-Field model of the BZ reaction with rate constants and residence time (2.17×10^{-3} s) the same as in ref. 12, and concentrations as in Fig. 1



except [malonic acid] = 0.24 M. Inset shows a 40-fold enlargement of map around fixed point (right) and shifted map (left) resulting from a 0.2% change in the flow rate of the bromate and cerium reactant stream. The system at state A_n is directed to A_s on the next return by varying μ according to equation (1) to shift map^{7,8}.

perturbation to the bifurcation parameter according to the difference between the system state A_n and the fixed point A_s

$$\Delta\mu = (A_n - A_s)/g \quad (1)$$

where g is a constant. The current amplitude A_n is measured and with the value of A_s obtained from the map, the value of $\Delta\mu$ necessary for the fixed point to be targeted is calculated. The value of the proportionality constant g can be determined by measuring the horizontal distance between two maps constructed at slightly different values of μ (refs 7, 8), as shown in Fig. 2*b*. Various period- k limit cycles can be similarly stabilized by using the corresponding values of A_s and g (obtained from

the appropriate maps of A_{n+k} against A_n) to determine $\Delta\mu$ in equation (1). The control algorithm may be implemented in several variations; for example, the perturbation determined every k th return may be applied for the entire period or for only a fraction of the period. In the stabilization of period-1 and period-2 shown in Fig. 1, the perturbation was applied for 15 s on each return.

Experimental fluctuations in the measured bromide potential result in significant scatter in the next-amplitude map, as shown in Fig. 2*a*. To reduce experimental noise, next-amplitude maps were used in the control algorithm rather than next-return maps; similar results were obtained with slightly more noise using

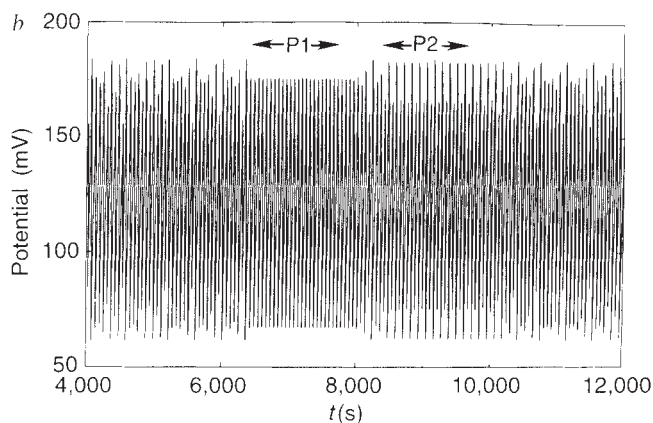
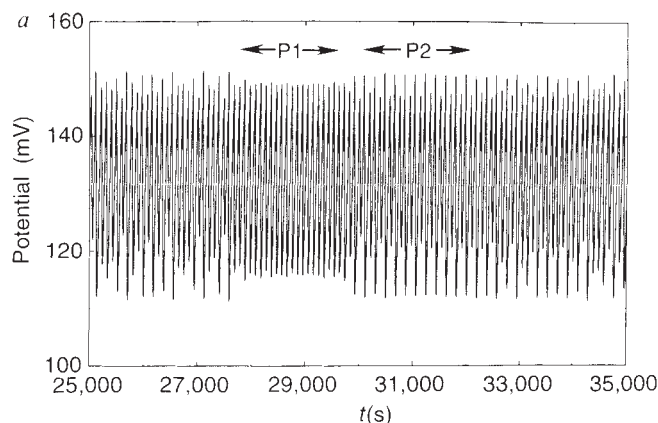


FIG. 3 *a*, Potential of bromide electrode as a function of time in BZ reaction with conditions given in Fig. 1. The control algorithm was switched on to stabilize period-1 at $t=27,800$ s and switched off at $t=29,500$ s, with the parameters corresponding to Fig. 1*a*. The parameters were changed at $t=30,000$ s to stabilize period-2, with values corresponding to Fig. 1*b*, until $t=32,100$ s when control was switched off. The control range was set at ± 3.0 mV for both period-1 and period-2. *b*, Oscillations in bromide potential calculated from Györgyi-Field¹² model showing chaos, stabilized period-1 and period-2, and the reappearance of chaos. Potential calculated from the Nernst equation and bromide concentration assuming an Ag/AgCl reference

electrode potential of 197 mV. Period-1 limit cycle stabilized by using $A_s=108.25$ mV and $g=-9.0 \times 10^4$ and period-2 limit cycle stabilized by using $A_s=90.235$ mV and $g=7.0 \times 10^4$ in equation (1). Controlling algorithm was applied on each return for 25 s and the control range was set at ± 5.0 mV. In both calculation and experiment, the flow rate of the bromate and cerium reactant stream was varied during control; a residence-time decrease of 1.0% therefore gives rise to an increase in these concentrations of 0.5% and a decrease in the malonic acid concentration of 1.0%. The typical residence-time variation during control was $\sim 1.0\%$ in experiment and $\sim 10^{-5}\%$ in calculation.

next-return maps. Although the experimental uncertainty seems to be comparable to that in previously reported maps for chaos in the BZ reaction^{9,10}, the scatter prevented the direct measurement of the proportionality constant g . The value of g was therefore estimated from the shift of A_s with variation of μ in the bifurcation diagram, and the final value was determined by adjustments around the initial estimate. Once the values for the fixed points and corresponding proportionality constants had been determined, the system could be switched between period-1 and period-2 behaviour and chaos by appropriately changing the values. Figure 3a shows the effect of the control algorithm, with the stabilization of the period-1 limit cycle followed by the period-2 limit cycle and the reappearance of chaotic behaviour when control was switched off. Transient aperiodic oscillations appear between period-1 and period-2 as the system leaves the period-1 orbit to arrive eventually at the control range of the period-2 orbit. When control was switched off, the chaotic behaviour was much the same as before the application of the feedback algorithm (Fig. 2a). Calculated behaviour using the Györgyi-Field¹² model is shown in Fig. 3b, with the stabilization of period-1 followed by period-2 and then a return to chaotic behaviour.

The stabilization of periodic orbits in chaotic systems by imposed feedback was first proposed by Ott, Grebogi and Yorke¹, and implications for biological self-regulation as well as practical uses such as convenient waveform generation have been pointed out¹⁻⁶. The quenching techniques of Sørensen and Hynne¹³⁻¹⁵ for targeting the unstable stationary state following a supercritical Hopf bifurcation are closely related to the OGY method. These authors have also successfully targeted the period-1 orbit in the BZ system following the initial period-doubling bifurcation, resulting in the transient appearance of these oscillations¹⁵. The map-based algorithm is more convenient than the OGY method for some experimental settings. (The OGY method has successfully been applied to stabilize period-1 in our BZ experiment, with results similar to those shown in Fig. 1a.) The map-based algorithm is especially useful in controlling high-frequency chaos, such as in laser² and diode³ systems. In these applications, as well as in our present experiments, it is advantageous to apply the controlling perturbation

for only a fraction of the oscillatory period. The system is attracted by the stable manifold of the unstable limit cycle during the perturbation-free fraction, thereby reducing the targeting error by ensuring that it resides effectively on the unstable manifold on its next return.

An important feature of both the OGY method and the map-based algorithm is that no knowledge of the underlying dynamics of a system (that is, the governing differential equations) is necessary for stabilizing any particular periodic orbit. This feature can be exploited to characterize experimentally the bifurcation behaviour of a chaotic (or periodic) system by tracking the unstable orbits as a bifurcation parameter is varied. The technique is similar to the computational algorithm AUTO¹⁶ for exploring the bifurcation behaviour of model systems.¹⁷ The tracking algorithm based on stabilizing periodic orbits, however, does not depend on model descriptions. We have made preliminary investigations of the BZ reaction using this technique with promising results; period-1 can easily be tracked after the first period-doubling bifurcation. □

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Rapid response of treeline vegetation and lakes to past climate warming

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FUTURE greenhouse warming is expected to be particularly pronounced in boreal regions¹, and consequent changes in vegetation in these regions may in turn affect global climate²⁻⁴. It is therefore important to establish how boreal ecosystems might respond to rapid changes in climate. Here we present palaeoecological evidence for changes in terrestrial vegetation and lake characteristics during an episode of climate warming that occurred between 5,000 and 4,000 years ago at the boreal treeline in central Canada. The initial transformation — from tundra to forest-tundra on land,

which coincided with increases in lake productivity, pH and ratio of inflow to evaporation — took only 150 years, which is roughly equivalent to the time period often used in modelling the response of boreal forests to climate warming^{5,6}. The timing of the treeline advance did not coincide with the maximum in high-latitude summer insolation predicted by Milankovitch theory⁷, suggesting that northern Canada experienced regionally asynchronous middle-to-late Holocene shifts in the summer position of the Arctic front. Such Holocene climate events may provide a better analogue for the impact of future global change on northern ecosystems than the transition from glacial to nonglacial conditions.

We obtained a 110-cm-long sediment core from Queen's Lake⁸ (unofficial name) and analysed it for fossil pollen, diatoms and sediment geochemistry in order to reconstruct and compare the changes in terrestrial and lake ecosystems that accompanied past climate warming at treeline (Fig. 1a-c). The lake is located at 64° 07' N, 110° 34' W, at an elevation of 480 m (Fig. 2). The surface area is ~50 ha and the maximum depth is 3.5 m. The region is rolling rockland composed of metamorphosed volcanics and intrusive granites. Cryic regosols and organic soils are found on discontinuous glacial overburden underlain by permafrost. Deglaciation commenced between 10,000 and 9,000 yr before present (BP)⁹. The lake is ~25 km north of the mapped limit of forest-tundra and lies on the boundary between the high subarctic and low arctic ecoclimatic regions¹⁰. The dominant vegetation is *Betula glandulosa* tundra. A few *Picea mariana* krummholz occur in the vicinity.

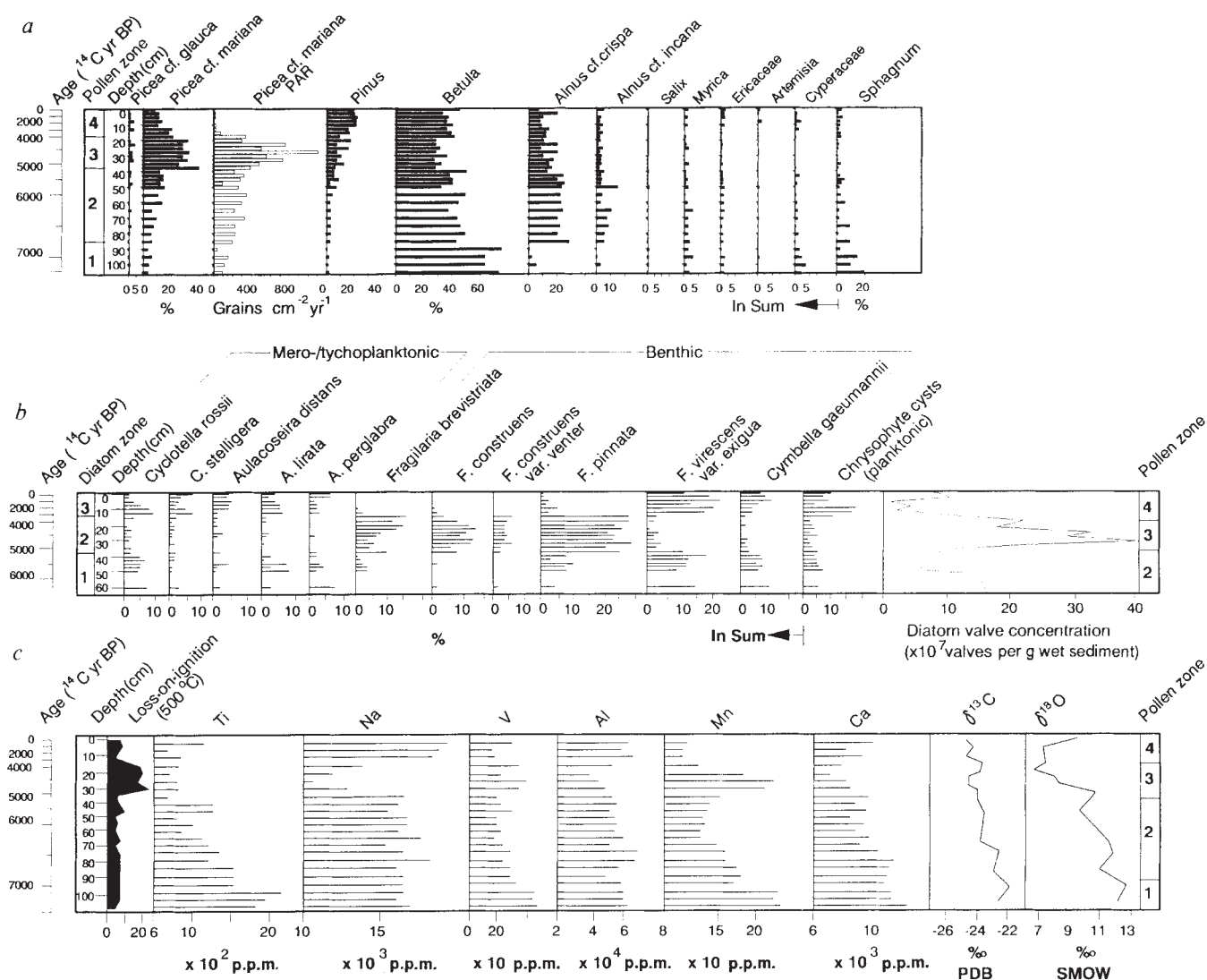


FIG. 1 Fossil pollen (a), diatom (b) and geochemical (c) stratigraphy of the Queen's Lake core. The entire core was sampled at 5-cm intervals for pollen and geochemical analyses. The upper portion was sampled at 2.5-cm intervals for pollen and diatom analyses. Sampling intervals were dictated by the quantity of sediment required and available for each technique and radiocarbon dating. Chronological control was provided by straight-line extrapolation between five radiocarbon dates (Table 1). The dates are internally consistent, have low standard errors and provide similar estimates of rates of sediment deposition and the timing of major vegetation changes as radiocarbon dates obtained from other lakes in the region (Table 1). Fossil pollen, diatoms and chrysophyte cysts were extracted and analysed using standard techniques²⁵⁻²⁷. The relative abundance of organic matter

was examined using loss-on-ignition (LOI)²⁸. The elemental geochemistry was determined using instrumental neutron activation analysis of bulk sediment^{29,30}. Carbon and oxygen isotope analyses were undertaken on cellulose extracted from the $<143 \mu\text{m}$ fraction of the sediments to estimate changes in the $\delta^{13}\text{C}$ of dissolved inorganic carbon (DIC) and the $\delta^{18}\text{O}$ of the lake water^{31,32}. Cellulose from such finely divided limnic organics appears to originate primarily from aquatic plants and algae, possibly reflecting the burial and preservation of zooplankton faecal pellets^{31,33}. Isotope ratios are expressed in conventional δ notation as deviations in parts per mil from the PDB and SMOW standards. Analytical uncertainties of $\pm 0.4\%$ apply for δ values.

At $\sim 5,000$ yr BP, there was a sharp increase in both the percentages and pollen accumulation rates (PARs) of *Picea cf. mariana* (Fig. 1a), suggesting a rapid increase in the density of trees. There was also a rapid decrease in pollen percentages of *Betula* at this time. However, the continued high abundance of *Betula* and *Alnus* pollen suggests that the site was occupied by forest-tundra rather than closed forest. A decrease in *Picea cf. mariana* pollen and an increase in *Betula* signals a return to tundra vegetation following 4,000 yr BP. The increasing abundance of *Pinus* pollen from 6,000 yr BP to the present represents the growth populations of *Pinus banksiana* located more than 100 km to the south⁸.

Before forest-tundra expansion at 5,000 yr BP, the diatom record was dominated by pennate benthic taxa such as *Fragilaria*

pinnata, *Fragilaria virescens* var. *exigua* and *Cymbella gaeumanni* (Fig. 1b). The latter two species are associated with slightly acidic waters. The most abundant centric diatoms are mero-tychoplanktonic species of the genera *Cyclotella* and *Aulacoseira*. The low diatom concentrations and dominance of acidophilous species indicate low lake productivity and pH. A sharp change in the lake at 5,000 yr BP is indicated by an abrupt increase in small benthic *Fragilaria* species, replacing the acidophilous benthic and mero-tychoplanktonic species as the most abundant forms. The cysts of planktonic chrysophytes declined in relative abundance, and total diatom concentrations reached a maximum. The high concentrations of diatoms coupled with the declines in acidophilous species indicate an increase in lake productivity during the forest-tundra period.

Invasion by spruce might promote lake acidification because surface water running through coniferous needle litter would be acidified¹¹. However, the results from Queen's Lake and studies of modern lakes¹² suggest that this is not always the case. Following 4,000 yr BP, the diatom record is marked by a return to conditions similar to those before 5,000 yr BP.

The loss-on-ignition (LOI) stratigraphy indicates a rapid increase in organic content at 5,000 yr BP and a decline following 4,000 yr BP (Fig. 1c). The abundance of crustal elements such as Ti, Na, Al and Ca declined between 5,000 and 4,000 yr BP (Fig. 1c). These results suggest increased lake productivity and/or decreased clastic erosion during the period of forest-tundra dominance. The increase in Mn between 5,000 and 4,000 yr BP is consistent with decreased lake water acidity and increased productivity¹³. It is not clear what might have caused the increase in V during this time.

The cellulose $\delta^{13}\text{C}$ in the sediments shifts from about -22% at the base of the core to -25% at the top (Fig. 1c). This decline probably reflects the increasing contribution of ^{13}C -deficient DIC, released by oxidation of organic matter accumulating in bottom sediments. A subdued excursion to higher $\delta^{13}\text{C}$ between $\sim 4,500$ and $3,500$ yr BP suggests a productivity-driven enrichment in DIC $\delta^{13}\text{C}$ consistent with the diatom, LOI and geochemical evidence for increased lake productivity during the forest-tundra period.

Lake water $\delta^{18}\text{O}$ values declined substantially during the shift from tundra to forest-tundra at 5,000 yr BP and then returned to intermediate values following 4,000 yr BP (Fig. 1c). This pattern probably reflects changes in the ratio of inflow to evaporation of the lake. By analogy with the interpretation of isotopes from lake carbonate¹⁴, covariance between $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ in the lower portion of the core suggests that the lake was initially a hydrologically closed basin. The decoupling of $\delta^{13}\text{C}$ and $\delta^{18}\text{O}$ following 6,000 yr BP suggests an opening of hydrological conditions because of increasing water inflow. The apparent acceleration of increase in the inflow:evaporation ratio at 5,000 yr BP probably reflects higher runoff and lower evaporation rates. This interpretation is consistent with data from modern lakes which indicate that water balance (inflow:evaporation ratio), rather than temperature-dependent variations in the $\delta^{18}\text{O}$ of local precipitation, determines the isotopic composition of lake waters in this region¹⁵. The interpretation is also supported by data indicating that tundra watersheds in central Canada experience higher evaporation rates than nearby forest-tundra sites¹⁶.

The changes in vegetation and lake characteristics at $\sim 5,000$ yr BP are not unique to Queen's Lake. We have obtained similar fossil pollen and LOI records from the three other treeline lakes that we have studied in central Canada (Fig. 2). The precise time when these records indicate maximum *Picea cf. mariana* pollen percentages and LOI values varies by several hundred years, and it is not possible to resolve whether these variations are real or simply noise associated with the radiocarbon dating of limnic sediments. These sites also record decreases in forest cover and lake productivity following 4,000 yr BP, although the rates and magnitudes of these decreases are more variable. The evidence of treeline advance at $\sim 5,000$ yr BP corresponds well with fossil pollen and palaeosol studies from more easterly sites in central Canada^{17,18}. In contrast, investigations in north-western Canada indicate a northern extension of forest by 9,000 yr BP and forest decline at 5,000 yr BP^{7,19}. The mid- to late Holocene timing of treeline change in central Canada cannot be attributed to the Milankovitch summer insolation maximum that has been used to explain early Holocene treeline advance in northwestern Canada⁷. It is possible that the environmental changes evident in central Canada reflect shifts in the summer position of the Arctic front caused by small changes in frontal wave characteristics^{18,19}. Such changes in geometry could cause treeline advance in central Canada with opposite or negligible impact in northwestern Canada¹⁹. A northward shift in central

TABLE 1 Radiocarbon dates (^{13}C -corrected) from lake sediment cores

Depth (cm)	Material	Age (yr BP $\pm 1\sigma$)	Laboratory number
<i>Queen's Lake</i>			
15–20	Organic sediment	$3,820 \pm 60$	WAT-1770
45–50	Organic sediment	$5,600 \pm 60$	WAT-1771
60–65	Organic sediment	$6,150 \pm 60$	WAT-1772
100–105	Organic sediment	$7,150 \pm 70$	WAT-1773
105	Twig	$7,470 \pm 80$	TO-827
<i>McMaster Lake</i>			
10–12	Organic sediment	$3,690 \pm 50$	TO-766
20–22	Organic sediment	$3,680 \pm 60$	TO-158
30–32	Organic sediment	$5,120 \pm 60$	TO-767
40–42	Organic sediment	$5,360 \pm 60$	TO-156
60–62	Organic sediment	$6,180 \pm 60$	TO-154
<i>Toronto Lake</i>			
35–40	Organic sediment	$1,760 \pm 90$	Beta-49705
80–85	Organic sediment and moss	$4,200 \pm 80$	Beta-53129
125–130	Organic sediment and moss	$5,460 \pm 90$	Beta-53130
155–160	Organic sediment	$7,040 \pm 120$	Beta-49708
<i>Waterloo Lake</i>			
28–31	Organic sediment	$4,030 \pm 50$	TO-3312
54–56	Organic sediment	$4,640 \pm 50$	TO-3311
61–63.5	Organic sediment	$5,300 \pm 50$	TO-3110
75–77	Moss	$7,640 \pm 100$	TO-3313

TO dates are based on accelerator mass spectrometry analysis and all others are conventional radiocarbon dates. Chronology for cores was derived using straight extrapolation between dates except at McMaster Lake where a line was fitted using the average extrapolated age provided by TO-766 and TO-158, and TO-767 and TO-156.

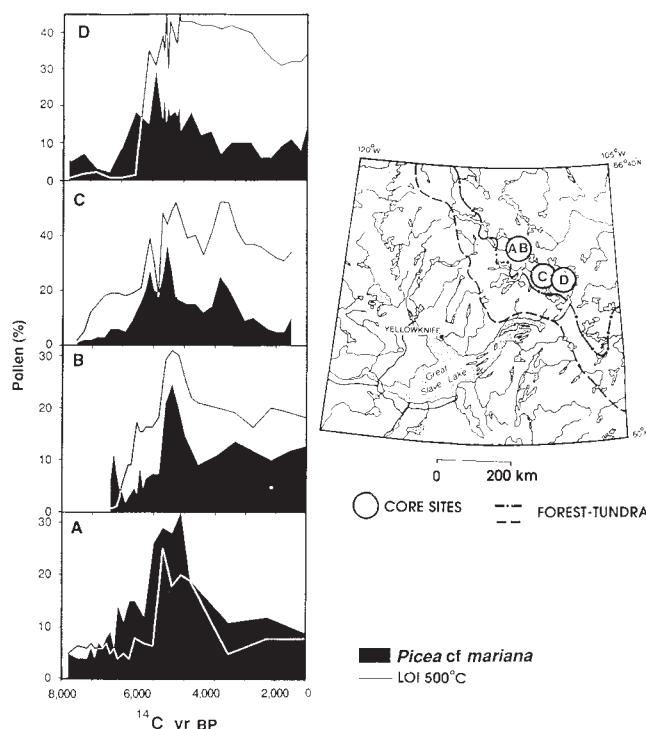


FIG. 2 Summary diagrams of *Picea cf. mariana* pollen percentages and LOI from the sediments of four small lakes located just north of the present forest-tundra in central Canada. A, Queen's Lake; B, McMaster Lake (unofficial name), $64^{\circ}08' \text{N}$, $110^{\circ}35' \text{W}$; C, Toronto Lake (unofficial name), $63^{\circ}43' \text{N}$, $109^{\circ}09' \text{W}$; D, Waterloo Lake (unofficial name), $63^{\circ}44' \text{N}$, $108^{\circ}06' \text{W}$. Radiocarbon dates used for chronological control are provided in Table 1.

Canada at 5,000 yr BP would produce warmer and moister summer conditions. A southward shift following 4,000 yr BP would cause a return to dominance by cool dry arctic air.

The earliest indications of the transition from tundra to forest-tundra vegetation and changes in lake characteristics are coincidental changes in the pollen and diatom percentages from the Queen's Lake core (Fig. 1). For the period 5,600–3,820 yr BP the time interval represented by 2.5 cm of deposition between each pollen and diatom sample is ~150 years. The shift in diatom flora from an acidophilous, low productivity assemblage to an assemblage reflecting higher pH and productivity occurs at exactly the same time as the shift in the pollen percentages. However, the shifts in total lake productivity as measured by diatom valve concentration, LOI and $\delta^{13}\text{C}$, and the geochemical and hydrological conditions of the lake as measured by elemental geochemistry and $\delta^{18}\text{O}$ were 150–300 years slower in registering a response to the shift from tundra to forest-tundra environmental conditions (Fig. 1). This suggests that the composition of the diatom flora adjusted quickly to temperature changes whereas adjustments of the total lake productivity and isotope balance took longer. Rapid change in small lakes, particularly in the composition of the diatom flora, is not surprising as short life cycles and widespread dispersal ability allow diatoms to respond quickly to changes in climate²⁰. Surprisingly, the record from Queen's Lake, and the information from the other sites, does not indicate a large time lag between changes in lake characteristics and changes in treeline vegetation (Figs 1 and 2). The rapid increase in *Picea mariana* at Queen's Lake may have been helped by its typical spatial distribution in central Canada, where scattered krummholz occur more than 100 km north of the mapped limits of forest-tundra. Intervals of mild climate as short as 10 years have been shown to initiate successful reproduction by treeline populations of *Picea* in eastern Canada²¹. Scattered trees that can rapidly produce viable seeds at the onset of climate warming provide ideal expansion foci which promote fast responses to favourable environmental changes²². The rapid changes in vegetation and lake characteristics at 5,000 yr BP may reflect rapid and synchronous climatic warming. It is also possible the nonlinear ecological responses caused by feedbacks between vegetation, permafrost, soils and boundary-layer climate could amplify rates of vegetation and lake ecosystem change once critical thresholds are crossed⁵.

Our evidence on the nature and rate of past environmental changes at treeline may help to predict the response of vegetation, hydrology and lake characteristics to future climate warming. These results show that for the central Canadian treeline, the 250-year time frame used in modelling the short-term response of northern boreal forests is reasonable^{5,6}. Palaeoecological investigations of *Pinus sylvestris* treeline in northern Scotland suggest similar rapid responses of vegetation and hydrology to shifts in the Azores high between 4,400 and 3,800 yr BP²³. The rapid change in the distribution of *Pinus sylvestris* may not have required the same spatial distribution of seed sources as we propose for *Picea mariana* in central Canada. Late Holocene episodes of climate change such as these in central Canada and Scotland may be better analogues for future global warming than is the change from glacial to nonglacial conditions, when the distribution of solar radiation and Earth boundary conditions were very different from today²⁴. It remains a concern that greenhouse warming may occur even faster than these Holocene events²³. □

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Origin of modal and rhythmic igneous layering by sedimentation in a convecting magma chamber

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EXPERIMENTAL investigations of convecting, particle-laden fluids show two regimes for convection driven by cooling from above¹. In very dilute suspensions, convection will maintain a homogeneous distribution of particles throughout the convecting layer provided that particle fall velocities are small compared with turbulent fluid velocities. Above a critical concentration, convection is unable to keep the particles suspended, so the particles settle, leaving behind a layer of convecting fluid virtually free of particles. Here we apply these results to cooling magma chambers, in which crystallization leads to an increase in suspended crystal content with time. Discrete sedimentation events are predicted each time the concentration exceeds the critical value. For common igneous minerals, critical concentrations are very small (typically 0.002–0.03 wt %) and layers of the order of centimetres to a few metres thick will result. Because minerals of different density and size have different critical concentrations and settling velocities, complex fluctuations in sedimentation rate and mineral proportions can occur in a multi-component melt. This may lead to either regular repetitive cycles or more complex fluctuations. The process is confined to low-viscosity magmas, such as basalts, in which the crystals are able to separate from the active thermal boundary layer during convection.

The concepts of crystal settling and convection in magma chambers have a long history^{2–6}. Wager and co-workers^{5,6} were struck by the strong resemblance of layered igneous rocks in the Skaergaard intrusion to clastic sedimentary rocks. Observations of graded bedding, trough structures and cross-layering

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suggested sedimentation from magmatic currents induced either by convection⁶ or by gravity currents⁷.

There have been doubts as to whether crystal settling can explain layering^{8,9}. Other mechanisms for layering have been recognized; these include *in situ* crystallization⁸, kinetic effects of crystal nucleation and growth^{10,11}, and double-diffusive layered convection^{8,9,12}. Experimental and theoretical studies^{1,13,14} of convecting suspensions heated from below show, however, that settling can occur at the base of a suspension even if the convection maintains a uniform concentration. Petrological observations¹⁵ show excess feldspar in many basaltic lavas which is most easily explained by crystal settling. The alternative layering models do not provide entirely convincing explanations of the basic 'cumulus' textures of layered rocks which are most simply interpreted as a framework of settled individual grains⁶. The oriented and dendritic morphologies due to growth *in situ* are less commonly observed than the apparent packstone texture. The more strikingly 'sedimentary' structures such as layers graded by grain size or density, and cross-layering, are also simply explained by sedimentation. Here we extend the results of experiments on convection in a suspension cooled from above¹. We propose a powerful mechanism for inducing the fluctuations in sedimentation rate and mineral proportions needed to cause much of the observed complexity in mesoscale modal and rhythmically layered rocks formed on the floors of magma chambers.

For our experiments we used a cubical tank of water seeded with silicon carbide grit of fairly uniform size, which was cooled uniformly from the top. This resulted in convection at high Rayleigh number ($Ra \approx 10^8$ – 10^9) with turbulent velocities at least two orders of magnitude greater than the settling velocities of the particles. For very dilute concentrations, the convection distributed the particles uniformly throughout the tank. Simultaneously, sedimentation occurred at the base, as in previous experiments¹³, so the mean concentration decreased with time. However, above a critical concentration a sharp descending interface was observed between a convecting upper clear region and a non-convecting lower region in which there was unimpeded sedimentation by Stokes law. Warmer fluid escaped through the interfacial region above the sedimenting layer and enhanced the convection driven by cooling at the roof. The lower-layer temperature remained uniform during sedimentation, confirming that it was stagnant.

The two-layer structure will be stable only if the density of the cool, clear upper layer is less than the bulk density of the sedimenting lower layer. Expressing this concept quantitatively and analytically solving the differential equations for the heat budget of the two layers¹, we showed rigorously that the two regimes are separated by a critical concentration

$$C_* = \frac{\rho_p}{\rho_p - \rho_0} \alpha (T_0 - T_u) \quad (1)$$

where ρ_0 is the density of the fluid at $T = T_0$, the constant temperature of the lower fluid layer, ρ_p is the particle density, α is the coefficient of thermal expansion of the fluid and T_u is the temperature of the upper fluid. The experimental observations¹ agree well with this criterion, especially when allowance is made for the transfer of small amounts of fine particles which are swept up into the upper convecting layer across the interface.

We now extend these concepts to combine the process of crystallization with the phenomena observed in the experiments, using inert particles to provide a general mechanism of layering (Fig. 1). We envisage a convecting magma chamber of height H , cooled from above. In addition there will be compositional convection from crystals at the floor⁹. Temperature decreases in the convecting interior. Suspended crystals form and are kept uniformly distributed within the interior. Concentration of crystals increases with time while some sedimentation from the suspension occurs on the floor¹³. The concentration of crystals reaches the critical value, as given by equation (1), and a discrete

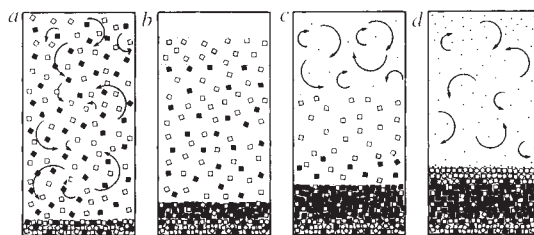


FIG. 1 Sequence of events in a magma chamber precipitating two phases of different size and density. *a*, Crystals are uniformly distributed through the chamber by convection. The interior cools, resulting in crystallization. Some sedimentation occurs at the chamber floor¹³ but crystal concentration increases with time. *b*, Critical concentration (equation (1)) is reached and the suspended crystals sediment in a stagnant layer in which convection is dampened. *c*, Clear, crystal-free convection layer forms above the sedimenting layer which forms a density-graded layer. *d*, Cycle begins again with formation of new crystals in the convecting chamber. Some of the lower-density crystals are shown swept up into the convecting layer.

sedimentation event follows. As the lower layer sediments, the upper layer continues to cool and crystallization begins again. The timescale for sedimentation is H/v_s where v_s is the Stokes free-fall velocity; the timescale for cooling is H^2/κ , where κ is the thermal diffusivity¹⁶. If the ratio of these timescales, κ/Hv_s , is small, as is typically the case, these processes can be considered to occur sequentially. In the simplest case of one phase, a repetitive sequence of layers should form on the floor of the chamber. If the sedimenting layer had a range of crystal sizes a graded bed could form, thereby providing an alternative mechanism to magmatic density currents⁷.

The thicknesses of the layers can be deduced from equation (1). Earlier studies, which equated the convective heat flux in the magma to the conductive heat flux through the solid overlying roof^{16,17}, indicate that the typical temperature differences driving convection in a magma chamber are 0.1–10 °C. Although these differences seem small, the resulting convective motions are sufficient to maintain crystals in suspension, except at the floor where sedimentation can occur¹³. Typical values of the relevant parameters are $\alpha = 5 \times 10^{-5} \text{ K}^{-1}$, $\rho_0 = 2,650 \text{ kg m}^{-3}$, $\rho_p = 3,300 \text{ kg m}^{-3}$ for olivine and $\rho_p = 2,700 \text{ kg m}^{-3}$ for plagioclase. For $T_0 - T_u \approx 0.1^\circ\text{C}$, the critical concentration is 0.0025 wt% for olivine and 0.027 wt% for plagioclase. For a chamber 1 km deep, the thickness of the sediment layer formed from such a sedimentation pulse would be 4 cm for olivine and 50 cm for plagioclase, assuming a packing fraction of 50%. For larger values of $T_0 - T_u$ the layer thicknesses will increase in proportion. Clearly there are other choices for the parameters, but for all realistic values the critical concentrations will be small, and the length scale of the individual layers will be a small fraction of the chamber depth in the range of a few centimetres to several metres.

The mechanism allows for great variation and complexity in magma chamber sedimentation. Each mineral phase will have a different critical concentration. Substantial fluctuations in sedimentation rate and proportions of each phase would be expected. We now discuss examples seen in the field which illustrate these concepts.

In the Rhum intrusion, centimetre- to metre-scale layering occurs in troctolites. Detailed stratigraphic logging^{18,19} reveals that the layering is largely caused by fluctuation in the size, shape and proportions of olivine surrounded by plagioclase. Average proportions of minerals are cotectic (70% plagioclase and 30% olivine), but these proportions fluctuate substantially from layer to layer. Although Rhum was an open-system chamber¹⁸, systematic changes in the mineral compositions in layered troctolites^{19,20,21} imply that the small-scale layering developed during periods of closed-system evolution. As olivine has a much lower critical concentration than plagioclase (see equation (1)), the scale of olivine layering should be smaller

than for plagioclase, as is observed. We envisage a convecting magma chamber precipitating olivine and plagioclase in cotectic proportions. When the concentration of crystals reaches a critical value, a sedimenting layer forms with the crystals settling at their Stokes fall velocities (Fig. 1). Olivine typically settles faster than plagioclase because of its greater density contrast with basaltic magma, so a layer of plagioclase is left behind (Fig. 1). If the plagioclase concentration is still less than critical for plagioclase alone, then the plagioclase would be mixed up into the 'clear layer' in the same way that in the experiments fine particles were swept up from the interfacial zone into the clear layer. Olivine and plagioclase continue to crystallize in the upper layer until critical concentrations are again reached and another pulse of sedimentation occurs. Eventually the critical concentration for plagioclase is achieved and the excess plagioclase is sedimented in a pulse generating anorthosite layers poor in olivine.

In the Skaergaard intrusion, one sees irregular modal layering, regular repetitions of graded beds (gravity stratification) and alternations of homogeneous gabbro cumulates and strongly density graded layers^{5,6,22}. We postulate that the alternation of homogeneous gabbro and graded layers is produced by a magma chamber that remains below the critical concentration for substantial periods of time and generates a steady accumulation of crystals on the floor in the cotectic proportions. A graded bed represents a pulse of sedimentation whenever the critical concentration is attained. In this case the crystals in the chamber settle out according to size and density.

Many complex interactions can take place with the co-precipitation of two or more minerals of different density. For example, as a plagioclase-rich layer sediments, fast-settling olivine formed in the overlying layer can fall through the interfacial zone and the excess density flux can overturn the layer. Nonlinear crystal growth and nucleation rates and the sedimentation velocities are coupled together with the cooling rate imposed by convection, magma viscosity and phase relationships. Complex fluctuations in the sedimentation rates and modal proportions are possible. It is not surprising that a wide range of patterns can develop, from the complex layering of Rhum troctolites to the regularity in parts of the Skaergaard intrusion.

The proposed mechanism requires that crystals formed in the thermal boundary layer at the roof of the chamber can escape by settling before the boundary layer becomes unstable locally. This will occur if the viscosity of the magma is low enough for crystals to escape from the boundary layer on the convective timescale rather than remaining to contribute to the density evolution of the boundary layer. If, instead, the viscosity is high and crystals are maintained in the boundary layer, the crystal content and density of the thermal boundary layer will always exceed the density of the underlying warmer suspension and equation (1) is not valid. We can estimate when equation (1) will be applicable when crystals are growing in the boundary layer by an approximate analysis of local conditions. The mean thickness, δ , of the thermal boundary layer is given by²³

$$\delta \approx (\mu \kappa R_c / \alpha \Delta T g \rho_0)^{1/3} \quad (2)$$

where R_c is the critical value of the Rayleigh number ($\sim 10^3$), μ is the magma viscosity, κ is the thermal diffusivity, α is the coefficient of thermal expansion, ΔT is the temperature difference driving convection, g is gravity and ρ_0 is the magma density. The timescale, τ , for the instability of the boundary layer to occur is given by the time taken for conduction to thicken the local boundary layer to this mean thickness

$$\tau \approx (\mu R_c / \alpha \Delta T \rho_0 g)^{2/3} \kappa^{-1/3} \quad (3)$$

The two different regimes can be separated by equating the settling time, ϕ/v_s to the convective boundary-layer timescale, τ . Using Stokes law for a crystal of diameter d and density contrast $\Delta\rho$ and equating (2) and (3), we find that the critical

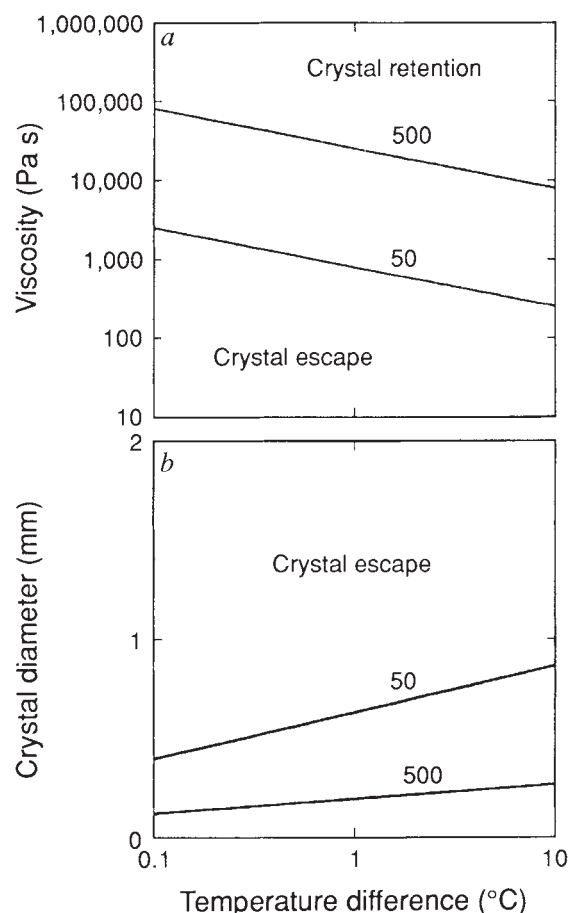


FIG. 2 *a*, Critical viscosity separating thermal boundary layers that retain crystals from those from which the crystals can escape is shown as a function of temperature difference driving convection. The lines show calculations for 5-mm-diameter crystals with density contrasts of 500 and 50 kg m⁻³ with the magma. Crystals can escape from sufficiently low-viscosity magmas before the thermal boundary layer destabilizes locally. *b*, Maximum crystal size that can be retained in the thermal boundary layer for a magma with viscosity 30 Pa s as a function of temperature difference driving convection. Calculations are shown for density differences between crystal and magma of 50 and 500 kg m⁻³. The calculations indicate that crystals larger than 1 mm can usually escape from thermal boundary layers before they become unstable locally.

viscosity, μ_c , below which equation (1) is valid is

$$\mu_c \approx \left(\frac{g \Delta \rho d^2}{18} \right)^{3/2} \left(\frac{R_c \mu \kappa}{\alpha \Delta T g \rho_0} \right)^{1/2} \kappa^{-1} \quad (4)$$

Figure 2*a* shows calculations of μ_c as a function of temperature differences in the range 0.1–10 °C for crystals of diameter 5 mm and density contrasts of 50 and 500 kg m⁻³. The temperature range covers typical values expected in magma chambers. Figure 2*b* shows the crystal size that must be exceeded for crystals to escape from the thermal boundary layer for a magma with viscosity of 30 Pa s, this being typical for basalt. We conclude that the mechanism will be effective in low-viscosity basic and intermediate magmas and can occur in basaltic magmas for crystals of diameter ≥ 1 mm. The absence of layering in rocks formed from viscous magmas is consistent with the rheological control implied by our analysis. Appropriate conditions for layering by the mechanism proposed here only occur when crystals escape from the unstable boundary layer or crystal nucleation occurs in the interior¹¹. □

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Carbon dioxide limitation of marine phytoplankton growth rates

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THE supply of dissolved inorganic carbon (DIC) is not considered to limit oceanic primary productivity¹, as its concentration in sea water exceeds that of other plant macronutrients such as nitrate and phosphate by two and three orders of magnitude, respectively. But the bulk of oceanic new production² and a major fraction of vertical carbon flux is mediated by a few diatom genera whose ability to use DIC components other than CO₂, which comprises <1% of total DIC³, is unknown⁴. Here we show that under optimal light and nutrient conditions, diatom growth rate can in fact be limited by the supply of CO₂. The doubling in surface water pCO₂ levels since the last glaciation from 180 to 355 p.p.m.^{5,6} could therefore have stimulated marine productivity, thereby increasing oceanic carbon sequestration by the biological pump.

Dissolved inorganic carbon in sea water exists in three interchangeable forms: CO₂, HCO₃⁻ and CO₃²⁻. Despite its low concentration (0.5%–1% of DIC, corresponding to 10–15 µM CO₂ at pH 8.2), CO₂ is the main source of inorganic carbon for phytoplankton growth in the natural environment. This is indicated both by phytoplanktonic stable carbon isotope compositions, which show a strong inverse relationship with ambient CO₂ concentrations⁷, and by high phytoplanktonic δ¹³C values during diatom blooms, which indicate limitation of the rate of photosynthesis by diffusive CO₂ transport⁸. Whereas some microalgae tested can use bicarbonate⁴, common marine diatoms use only dissolved CO₂ (ref. 9).

Uptake of inorganic carbon by a photosynthetically active cell leads to concentration gradients of CO₂, HCO₃⁻ and CO₃²⁻ in the surrounding medium which deviate from equilibrium. At steady state, CO₂ uptake is balanced by diffusion of CO₂ down the gradient to the cell surface and conversion of HCO₃⁻ to CO₂. Diffusional transport to the cell surface is dominated by molecular diffusion, because turbulent shear is insignificant at phytoplankton size scales that are small compared with the Kolmogorov length scale¹⁰. By combining the processes of CO₂ molecular diffusion and its conversion from bicarbonate¹¹, the transport equation for CO₂ reads

$$\frac{D}{r^2} \frac{d}{dr} \left(r^2 \frac{dc}{dr} \right) + k'(c_\infty - c) = 0 \quad (1)$$

where D is the diffusivity of CO₂, c is the concentration as a function of the radial distance r from the centre of the cell, c_∞ is the concentration of the bulk medium, k' the rate constant for conversion of HCO₃⁻ to CO₂ ($k' = k_1[\text{OH}^-] + k_2$, where k_1

is the rate constant for direct formation of CO₂ from bicarbonate, $[\text{OH}^-]$ the hydroxyl ion concentration, and k_2 the rate constant for the formation of CO₂ from H₂CO₃). With $c(r \rightarrow \infty) = c_\infty$ and $c(r = a) = c_a$, a being the cell radius, integration of equation (1) yields

$$\frac{c - c_\infty}{c_a - c_\infty} = \frac{a}{r} \exp\left(\frac{a - r}{a_k}\right) \quad (2)$$

where $a_k = \sqrt{D/k'}$. The total flux of CO₂ to the cell, Q_a , is then given by

$$Q_a = -4\pi a^2 D \frac{dc}{dr} = 4\pi a D \left(1 + \frac{a}{a_k}\right) (c_\infty - c_a) \quad (3)$$

In equation (3), the quotient a/a_k represents the portion of Q_a contributed by conversion of CO₂ from HCO₃⁻. This portion can be calculated for any given cell size, a , from the a_k values plotted in Fig. 1 as a function of pH and temperature. For a typical diatom, cell radius $a = 15$ µm, the fraction of CO₂ supplied by conversion from HCO₃⁻ would lie between 15/300 (5%) and 15/800 (~2%) of the total CO₂ supply. Thus, as for other nutrients, the transport of CO₂ to the cell surface is predominantly by way of molecular diffusion.

Comparison of the CO₂ flux to the cell surface with the flux of nitrate and phosphate (calculated from equation (3)) yields a flux ratio of C/N/P which deviates significantly from the Redfield ratio. At nutrient concentrations typical of conditions at the onset of high-latitude phytoplankton spring blooms and

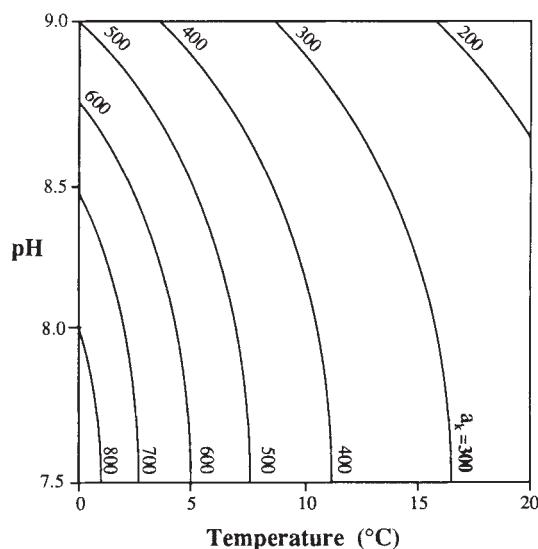


FIG. 1 Value of a_k (in µm) as function of pH and temperature; a/a_k provides the relative CO₂ supply to the cell from conversion of HCO₃⁻ to CO₂. HCO₃⁻ conversion becomes significant only for large cell sizes ($a > 50$ µm) at high pH and temperature. At pH and temperature ranges generally observed in the ocean (pH 7.8–8.5; 0–20 °C), conversion of HCO₃⁻ to CO₂ contributes less than one-tenth of the total CO₂ supply to a typical phytoplankton cell ($a = 5$ –30 µm).

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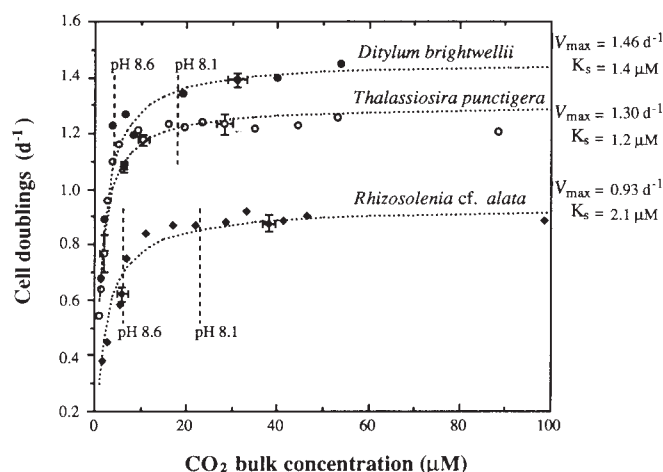


FIG. 2 Specific growth rates as a function of CO₂ concentration. Cells were grown in nutrient-enriched (at 1/10 of the concentrations given in ref. 24), 0.2-μm filtered natural sea water at 120 μE m⁻² s⁻¹ light intensity and *T* = 17 °C (*D. brightwellii* and *T. punctigera*) or 5 °C (*R. cf. alata*). Each data point represents the mean specific growth rate estimated from daily cell counts over a 5–6 day period. CO₂ concentrations were adjusted by keeping pH constant at values between 7.5 (~95 μM CO₂) and 9.3 (~0.8 μM CO₂). DIC and CO₂ concentrations were calculated from measurements of pH, alkalinity, salinity and temperature²⁵. The maximum number of cells at the end of the experiments was generally below 3 × 10⁶ cells l⁻¹, which corresponds to a reduction in total DIC (C_i) of less than 10% and an increase in alkalinity, due to nitrate uptake by the algae, of less than 1.5%. Error bars = ±1 s.d., *n* = 3. V_{max} and K_s values were derived by feeding the data into equation (4). Vertical dashed lines indicate pH values.

blooms in upwelling regimes¹² (CO₂, nitrate and phosphate concentrations of 10–15 μM, 15–35 μM and 1–2 μM, respectively), the ratio of the maximum potential CO₂/NO₃⁻/PO₄³⁻ fluxes is ~28/38/1 (assuming $D_{\text{CO}_2} = 1.60 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (ref. 13), $D_{\text{NO}_3} = 1.65 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (ref. 14), $D_{\text{PO}_4} = 0.68 \times 10^{-9} \text{ m}^2 \text{ s}^{-1}$ (ref. 14), and equal ratios of c_a/c_∞ for the three nutrients). Comparing this ratio to the C/N/P requirement of the cell as given by the Redfield ratio¹⁵, 106/16/1, reveals that CO₂ is in fact the nutrient in shortest supply.

For a photosynthesizing algal cell to compensate for the deficiency in the supply of CO₂ relative to other nutrients would require a concentration gradient to be maintained between the bulk medium (c_∞) and its cell surface (c_a) which is considerably steeper for CO₂ than for the other nutrients; c_a can be obtained by combining the nutrient flux model given by equation (3) with the Michaelis-Menten or Monod uptake model¹⁶ which describes the relationship between nutrient uptake rate and nutrient concentration

$$Q_a = 4\pi a D \left(1 + \frac{a}{a_k} \right) (c_\infty - c_a) = \frac{V_{\max} c_a}{K_s + c_a} = Q_M \quad (4)$$

where $V_{\max}$ is the maximum uptake rate and K_s the half-saturation constant. Solving equation (4) for c_a yields $c_a(a) = -0.5\alpha + \sqrt{0.25\alpha^2 + \beta}$, with $\alpha = K_s - c_\infty + V_{\max}/[4\pi D(1 + a/a_k)]$, and $\beta = K_s c_\infty$. For nutrients other than CO₂, $a/a_k = 0$.

Assuming $V_{\max}$ and K_s values typical of marine phytoplankton ($V_{\max} = 1.5$ doublings per day (ref. 17); $K_{s(\text{CO}_2)} = 0.5 \mu\text{M}$ (ref. 18); $K_{s(\text{NO}_3)} = 0.5 \mu\text{M}$ (ref. 19), a diatom cell of radius $a = 30 \mu\text{m}$ growing under pre-bloom nutrient concentrations (see above) and maintaining a CO₂/NO₃⁻ flux ratio of 106/16 would generate cell-surface CO₂ and nitrate concentrations of $c_a(\text{CO}_2) \approx 32\%$ and $c_a(\text{NO}_3) \approx 96\%$ of their respective bulk concentrations. Based on the Monod uptake model, a $c_a(\text{NO}_3)$ of 96% of the bulk concentration reduces the nitrate-specific growth rate to ~98% of $V_{\max}$, whereas at $c_a(\text{CO}_2) = 32\%$, the carbon-specific growth rate is reduced to 86.5% of $V_{\max}$. Thus, even if a CO₂/NO₃⁻ flux equivalent to the Redfield C/N ratio could be maintained, growth rate of our model diatom would be limited by CO₂.

To test the model predictions, specific growth rates of unialgal cultures of two temperate (*Ditylum brightwellii* and *Thalassiosira punctigera*) and one polar diatom species (*Rhizosolenia cf. alata*) were determined at constant CO₂ concentrations ranging between 0.8 and 95 μM. At [CO₂] < 10–15 μM, cell division rates of the three species decreased with decreasing CO₂ levels (Fig. 2). Above this level, growth rate was unaffected by CO₂ concentration. Similar results were obtained in experiments in which pH was allowed to increase gradually with photosynthetic carbon fixation. DIC uptake rates calculated from the change in pH over time correlated positively with CO₂ at concentrations below 10–15 μM (Fig. 3). For the three species tested, DIC

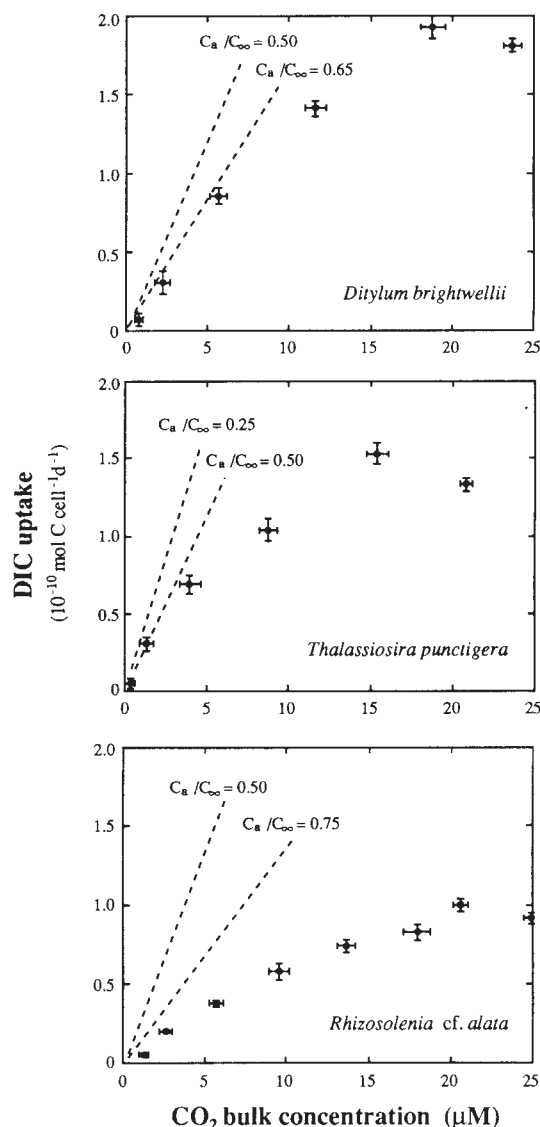


FIG. 3 DIC uptake as a function of CO₂ concentration. Cells were grown in closed (no air exchange) 100-ml Duran glass bottles under light, temperature and nutrient conditions as in the other experiments. DIC uptake rates were calculated from daily changes in pH. Data represent means and standard deviations of 4 replicate experiments. Dashed lines indicate theoretical maximum CO₂ fluxes as calculated from equation (3) for different concentration gradients (c_a/c_∞) between the cell surface (c_a) and the bulk medium (c_∞). Uptake rates reverted to initial values after bubbling the cultures with CO₂ gas for 15–20 seconds.

uptake rates always remained lower than theoretical CO₂ fluxes calculated for cell-surface CO₂ concentrations above 50% of the bulk concentration ($c_a/c_\infty > 0.5$), suggesting that bicarbonate utilization as a mechanism to compensate CO₂ limitation is insignificant.

The experimental evidence corroborates the key result of the model that, under conditions giving rise to phytoplankton blooms (temporary nutrient excess), the growth rate of marine diatoms can in fact be limited by the CO₂ supply to the cell surface, particularly during intense blooms when $p\text{CO}_2$ values in the surface layer decrease drastically²⁰. The increase in atmospheric CO₂ levels between glacial and interglacial periods from 180 to 280 p.p.m.^{5,6} would raise CO₂ concentrations in air-equilibrated surface water from 7.9 to 12.3 μM (at 10 °C and 35‰ S). Assuming a nitrate uptake of 15 μM and a C/N ratio of 6.6, a bloom developing under glacial CO₂ conditions would reduce ambient CO₂ concentrations from 7.9 to 4.9 μM leading to severe CO₂ limitation of the cells (Fig. 2). Its interglacial counterpart would reduce ambient CO₂ from 12.3 to 7.3 μM with the cells still experiencing CO₂ limitation. The recent anthropogenically mediated increase in CO₂ levels from 280 to 355 p.p.m. would alleviate CO₂ limitation, however, as concentrations would now decline from 15.6 μM in air-equilibrated surface water to 9.0 μM at the end of the bloom.

Because of the large pool of DIC in sea water, the CO₂ supply can limit growth rate but not the total biomass attained by a diatom bloom after exhaustion of nitrate and phosphate. But as the rate of biomass build-up by a bloom is a function of both algal growth rate and loss terms, in particular grazing, the biomass attained during the bloom is directly proportional to the growth rate. Because the magnitude of particle sinking is a function of algal biomass concentration²¹, higher algal growth rates will enhance vertical carbon flux and increase remineraliz-

ation depth. Enhanced output by the biological pump would ultimately affect the vertical distribution of inorganic nutrients in the ocean, transferring nutrients from the surface (that is, above the permanent pycnocline; see ref. 21) to the deep ocean. It follows that, contrary to current opinion^{22,23}, the ocean's capacity to sequester carbon by the biological pump may well be positively correlated to atmospheric CO₂ concentrations. □

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Sources of variations in patterns of plant–insect association

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MODELS of evolution in parasite–host or predator–prey systems assume that particular pairs of species either always interact or never interact¹. But, in nature, a particular parasite species may interact with a particular host species at some times (or in some places) but not at others, even if host availability is not variable. These differences could stem either from variation among conspecific hosts in their resistance to parasitism or from variation among conspecific parasites in their tendency to attack particular host species. We report here that both of these effects exist, that they are probably due to genetic variation both in parasites and in hosts, and that they interact to produce spatial variation in ecological relationships. We studied three potentially interacting species, a herbivorous insect and two of its potential host plants, at each of two sites. At one site, a plant species was avoided by the insects because of a combination of local insect preference and local plant resistance. This result shows that the diet is a property neither of the parasite nor of the host, but of the parasite–host interaction.

Conspecific insects may select different host plant species from apparently similar arrays of potential hosts^{2–5}. An entomocentric approach to understanding this variation has shown that host preferences of these insects may vary among individuals or populations, and cause them to select different diets^{5–9}. Conversely, a phytocentric approach has shown that

TABLE 1 Numbers of insects preferring *P. rydbergii* or *C. parviflora*

	<i>P. rydbergii</i>	<i>C. parviflora</i>
(a) Field trials, plants undisturbed		
Frenchman insects, tested at Frenchman	23	0
Sonora insects, tested at Sonora	0	11
	$P = 3.5 \times 10^{-9}$	
(b) Field-caught insects tested on potted plants		
Frenchman insects, tested on		
Sonora plants	28	0
Sonora insects, tested on Sonora plants	1	6
	$P = 4.3 \times 10^{-6}$	
(c) Lab-raised insects tested on potted plants		
Frenchman insects, tested on		
Sonora plants	17	0
Sonora insects, tested on Sonora plants	0	9
	$P = 3.2 \times 10^{-7}$	

Probabilities calculated by Fisher's exact test. All potted plants were growing in soil from their site of origin. As far as possible, within each set of trials, all insects were tested on the same individual plants. Thus, each experiment estimated variation among insects by controlling for variation among plants. Line 2 of *a* is taken from ref. 12. We used a manipulative technique, justified elsewhere¹², that involves placing insects on test plants in alternation, at 10-min intervals. If the insect attempts to lay eggs, it is deemed to accept the plant; if not, the plant is rejected. When an acceptance is recorded, the insect is not allowed to oviposit, and is then tested on the second plant. If the second plant is rejected, a preference for the first plant is recorded. This technique is based on the finding that different plants become acceptable to *E. editha* at different times, but that once a plant is accepted, it remains acceptable until oviposition occurs. The insects show no effects of experience in their oviposition choice behaviour.

conspecific plants may be attacked by different insect species, and that genetic variation among these plants may influence the identities of the insect species that associate with them^{10,11}. Here, we integrate these two approaches by asking whether an observed case of geographical variation of diet is generated by variation of insect traits, plant traits or both.

Our study organisms were the nymphaline butterfly *Euphydryas editha* and two of its host species, *Penstemon rydbergii* and *Collinsia parviflora*, both in the Scrophulariaceae. Our two study sites, at Frenchman Lake (Plumas County, California) and Sonora Junction (Mono County, California) were about 150 km apart and had almost identical vegetation. At both sites *C. parviflora* has been more abundant than *P. rydbergii* in each of the years from 1988 to 1991. At Sonora, *P. rydbergii* was not used by the butterfly at all from 1986 to 1989, and a single egg cluster was found on it in 1990. Most eggs were laid on *C. parviflora*¹² and butterflies tested on plants *in situ* preferred this species (Table 1a). At Frenchman Lake, *P. rydbergii* was preferred by insects tested *in situ* (Table 1a), and received 20 of 23 egg clusters found in a field census. A single cluster was found on *C. parviflora*. The proximate causes of this diet difference among sites could lie in variation of either insect and/or plant traits. We did experiments to elucidate these causes.

To ask whether insect preferences for the two plant species were different between the sites, we controlled for effects of plant origin by testing insects from the two populations on potted Sonora plants. Insects from the two populations differed in preference, with each tending to prefer the host species that it used naturally (Table 1b). Next, we asked whether conspecific plant populations differed in resistance, estimated from their acceptability to ovipositing butterflies. Frenchman *P. rydbergii* were more acceptable than those from Sonora to insects from both origins (Table 2). *C. parviflora* from the two sites were not significantly different.

Both insect preference and host acceptability differed between sites. Did both these effects contribute to the observed variation of diet? To ascertain the role of plant acceptability, we asked whether insects would rank the two plant species differently, depending on the site of origin of the plants. Sonora insects showed a significantly increased tendency to prefer *P. rydbergii*

TABLE 2 Numbers of independent plant pairs in which a *P. rydbergii* from Frenchman Lake (FM) was either more or less acceptable than a *P. rydbergii* from Sonora Junction (SJ)

	FM > SJ	FM = SJ	FM < SJ	
Transplanted plants tested with FM insects	11	0	0	$P=0.0005$
Transplanted plants tested with SJ insects	9	3	0	$P=0.002$
Plants grown from seed, tested with FM insects	9	3	0	$P=0.002$
Plants grown from seed, tested with SJ insects	9	2	0	$P=0.002$

Plants were either transplanted or grown from seed in soil from their site of origin, as indicated. Here, we are interested in the response of each population of insects to both populations of *P. rydbergii*. Our null hypothesis is that the two populations are not different in their acceptability to the butterflies. Each data point should make an independent contribution to testing this hypothesis, so we used each insect only once, and each plant pair only once. Specifically, each *P. rydbergii* plant from Sonora was paired with a phenologically similar *P. rydbergii* from Frenchman. Each plant pair was offered to a single butterfly, and the origin of the more acceptable individual was recorded. Then both the plant pair and the insect were discarded and a fresh pair was tested with a fresh insect. The data consist of the number of plant pairs in which the Frenchman plant was ranked more acceptable than the Sonora plant, and the number of pairs in which the reverse result was obtained. Probabilities by binomial test, with the expectation of equal probability of FM < SJ and SJ < FM. The category FM = SJ was ignored in calculating significance.

TABLE 3 Rank order of plant species by insects offered local or foreign potted plants

Numbers of pairs in which:	<i>P. rydbergii</i> preferred	<i>C. parviflora</i> preferred
(a) Sonora plants, tested with Sonora insects	1	15
Frenchman plants, tested with Sonora insects	8	10
	$P=0.02$	
(b) Frenchman plants, tested with Frenchman insects	26	0
Sonora plants, tested with Frenchman insects	22	0
	$P=1.0$	

Each *P. rydbergii* was paired with a *C. parviflora* from the same population. We offered each insect two plant pairs, one pair from Sonora and one from Frenchman. Each pair consisted of one member of each species. The experimental design was described in the caption to Table 2. Thus, when we had obtained a preference, we discarded both the plant pairs and the insect, then selected two new pairs and a new insect. Probabilities calculated by Fisher's exact test.

over *C. parviflora* when offered plants from the foreign site (Table 3a). Thus, the intersite variation of plant acceptability played a role in excluding *P. rydbergii* from the diet of Sonora insects. The aversion of Sonora butterflies to *P. rydbergii* in general also contributed to this exclusion because Frenchman butterflies preferred *P. rydbergii* over *C. parviflora* even when tested on Sonora plants (Table 3b). These results show that interpopulation variation of a plant trait, acceptability, interacted with that of an insect trait, preference, to generate the geographical variation of diet, and to remove the insect from significant ecological interaction with *P. rydbergii* at Sonora.

Evidence from our study populations strongly suggests that this variation has a genetic basis: for both plants and insects, the differences between our study populations were maintained in laboratory culture through one complete generation. When *P. rydbergii* from the two sites were grown from seed in a reciprocal transplant design, the difference in acceptability between plants from the two populations was preserved, irrespective of soil type (Table 4). Likewise, when insects from Frenchman and Sonora were raised from the egg in captivity on *C. parviflora*, the adults retained the preferences characteristic of their populations (Table 1c). Although it is possible that environmentally caused differences may persist without diminution through a generation in the laboratory, we consider this unlikely. The probability of strong effects of maternal environment in *P. rydbergii* is low, because the seeds are extremely small (3×10^{-5} g). In studies of other *E. editha* populations, reciprocal crosses have given unequivocal evidence that interpopulation variation of preference was genetically based⁸.

This study is to our knowledge the first to show that ecological interactions among species can be made and broken by the combined effects of variation within both members of a species

TABLE 4 Numbers of independent plant pairs in which a *P. rydbergii* from Frenchman Lake (FM) was either more or less acceptable than a *P. rydbergii* from Sonora Junction (SJ)

	FM < SJ	FM = SJ	FM > SJ	
Plants grown from seed in FM soil	10	0	0	$P=0.001$
Plants grown from seed in SJ soil	12	2	1	$P=0.005$

Design as in Table 2. Each butterfly was used only once. Butterflies were all from Frenchman. Probabilities by binomial test, with the expectation of equal probability of FM < SJ and SJ > FM. The category FM = SJ was ignored in calculating significance.

pair. Other work has shown that the nature of interactions, rather than their existence, may be influenced by such variation^{13–18}. For example, a decline in the severity of the disease myxomatosis resulted from the combined evolution of increased resistance in rabbits and reduced virulence in the myxoma virus¹⁹. In this case the symptoms of the disease comprised the trait of the interaction, and the variation within species was over time rather than over space, as in our example.

We make no implication that variation in plant acceptability is an evolutionary response to attack by the insects we have studied. Nor do we know whether variation of insect preference is an evolutionary response to intrinsic plant quality rather than to other ecological factors²⁰. But we can conclude that the evolution of diet must emerge from separate evolution of plant and insect traits. Models of diet evolution should treat diet as a trait of the interaction among species, rather than as a property of either parasites or hosts. □

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Vertical disparities, differential perspective and binocular stereopsis

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TO calculate the depth difference between a pair of points on a three-dimensional surface from binocular disparities, it is necessary to know the absolute distance to the surface^{1,2}. Traditionally, it has been assumed that this information is derived from non-visual sources such as the vergence angle of the eyes^{3,4}. It has been shown^{5,6} that the horizontal gradient of vertical disparity between the images in the two eyes also contains information about the fixation distance^{7–9}. Recent results^{10,11}, however, indicated that manipulations of the vertical disparity gradient have no effect on either the perceived shape or the perceived depth of surfaces defined by horizontal disparities. Following the reasoning of Longuet-Higgins¹² and Tyler¹³, we suggest that vertical disparities are best understood as a consequence of perspective viewing from two different vantage points and the results we report here show that the human visual system is able to exploit vertical disparities and use them to scale the perceived depth and size of stereoscopic surfaces, if the field of view is sufficiently large.

Figure 1a and b depicts, from behind, a pair of eyes viewing a rectangular pattern lying in a fronto-parallel plane which is either close to the observer (a) or far away (b). On the retina of the left eye in 1a, the (inverted) image of the left-hand edge is shown as taller than that of the right-hand edge, to reflect the fact that the left-hand edge is nearer that eye, and therefore subtends, at its nodal point, a distinctly larger angle than the right-hand edge. For the right eye the converse is true. For a distant surface of the same angular extent (Fig. 1b), the difference in angular size of the two edges becomes vanishingly small. It follows that the angle a vertical edge subtends at the left eye divided by the angle it subtends at the right eye is greater than 1 for the left-hand edge of the pattern, but is less than 1 for the right-hand edge. We refer to this ratio, or rather the ratio of the tangents of the two angles, as the vertical size ratio (VSR). It is the ratio of the inverse distances of the edge from the two eyes, and is therefore a well defined function of horizontal eccentricity, independent of vertical elevation. The first equation

of Fig. 2 expresses the VSR as a function of e , the horizontal eccentricity; D , the distance of the plane; and IOD, the interocular distance. Its dependence on e and D (for IOD = 6.5 cm) is illustrated on the left-hand side of Fig. 2 and may be described as a manifestation of differential vertical perspective. It is clear that (as Mayhew and Longuet-Higgins showed in the general case⁵) the sensitivity of VSR to e is a strong cue to the value of D , the observer's distance from the surface¹⁴.

Mayhew and Longuet-Higgins' theory established that binocular viewing of an extended surface introduces differential vertical perspective cues that vary with the distance to the surface¹⁵. Figure 1 shows that the binocular viewing of a fronto-parallel surface close to the observer also generates a gradient in the horizontal size ratio (HSR) as a function of eccentricity, which we refer to as differential horizontal perspective. As with the differential vertical perspective cue, the horizontal perspective cue is a consequence of the perspective viewing of a surface from two spatially separate vantage points and it also varies with the absolute distance to the surface (Fig. 2b). The distinction between the two cues is important because manipulating vertical disparities in the way described by Sobel and Collett¹¹, for example, has the effect of creating both differential vertical and differential horizontal perspective cues. Consequently a positive result, if it had been obtained, could not be attributed unambiguously to the presence of the vertical perspective cues.

Figure 2a and b shows that the binocular size ratios of a small element seen by the two eyes increase with eccentricity from the median plane and reach maxima at eccentricities of ± 45 degrees. Consequently, the failure of both Cumming *et al.*¹⁰ and Sobel and Collett¹¹ to find an effect of vertical disparities might have been due to the relatively small angular extents (8 – 11° and 30°) of the stereoscopic surfaces used in their experiments. To maximize the chances of showing the effects of the differential perspective cues in the experiments reported here, observers were presented with large-field ($80 \times 70^\circ$) displays. To test whether human observers use the differential perspective cues to scale perceived depth, observers were asked to make judgements about the amount of perceived peak-to-trough depth of sinusoidal corrugations defined by horizontal disparities. The differential perspective cues (either vertical or horizontal, or both together) were presented on different trials and were appropriate to viewing a surface at either 28 cm or infinity.

The results show that a surface containing both differential vertical and differential horizontal perspective cues appropriate to viewing the surface at infinity was judged to have more peak-to-trough depth than the equivalent surface with

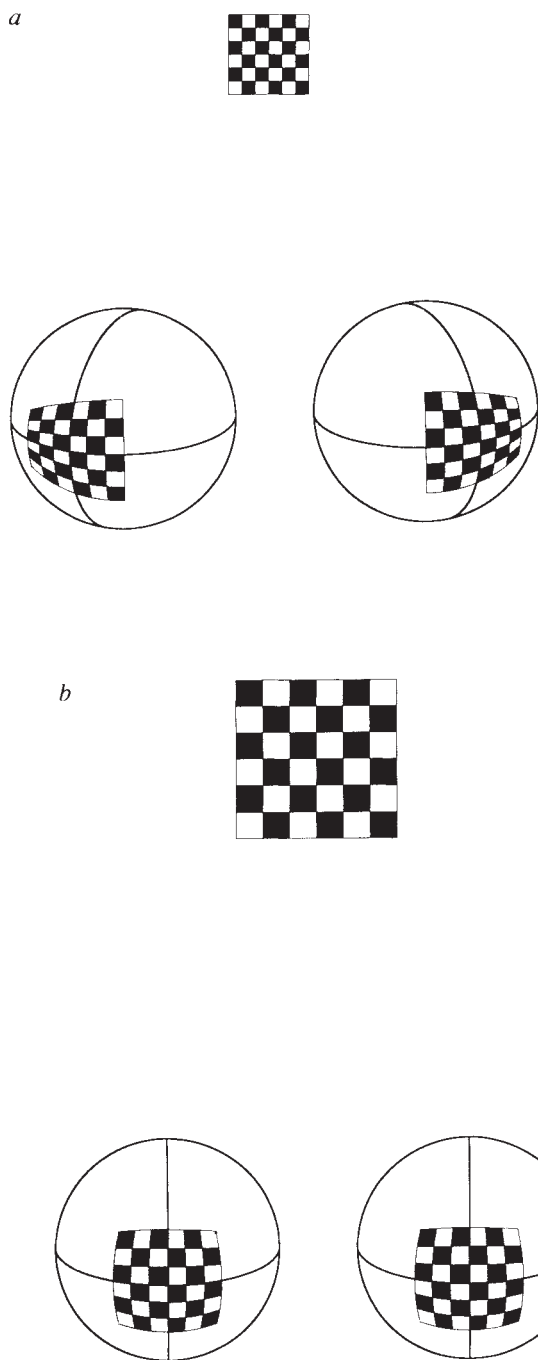


FIG. 1 The images created by the binocular viewing of a flat, rectangular, fronto-parallel surface either close to the observer (*a*) or far away (*b*), but of the same overall angular extent. When the surface is close, there are opposite gradients in the vertical angular subtense of individual chequerboard squares (from left to right) to the two eyes, because the left-hand edge of the surface is closer than the right-hand edge, at the left eye, and vice versa at the right eye. This effect can be understood intuitively as a consequence of the perspective viewing of a surface from two slightly different positions. The gradient in the vertical size ratio is referred to as differential vertical perspective. Panel *a* also shows that there are opposite gradients in the horizontal angular subtense of individual chequerboard squares (from left to right) to the two eyes, again as a result of the unequal distances to the two eyes. The gradient in the horizontal size ratio is referred to as differential horizontal perspective. As the surface approaches infinity the distances from the two eyes to all squares on the surface become more similar and thus the perspective views become identical.

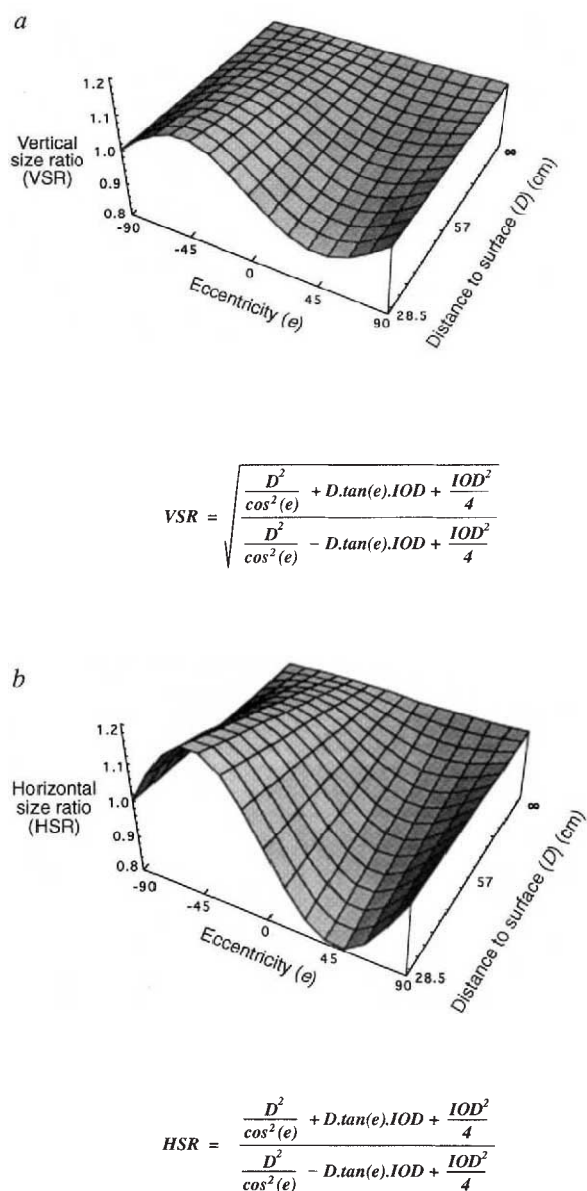
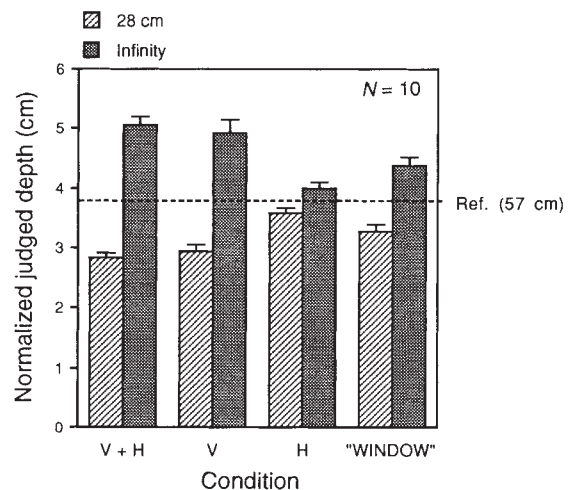


FIG. 2 *a*, How the vertical size ratio (VSR) of a small element on a fronto-parallel surface (measured in angular units) varies with cyclopean eccentricity, e , and the normal distance to the surface, D . The equation used to generate the graph is shown underneath, with the interocular distance IOD set to 6.5 cm. As the fronto-parallel surface approaches infinity, the vertical size ratio of a small element on that surface will always be close to 1.0 for all eccentricities (because the distances from the two eyes are nearly equal). *b*, How the horizontal size ratio (HSR) of a small element on a fronto-parallel surface (measured in angular units) varies with e , and D . The equation used to generate the graph is shown. The HSR will also be close to 1 for all eccentricities as the surface approaches infinity (*b*). For the fronto-parallel surface illustrated here, there is a unique relationship between the values of the VSR and the HSR for all distances and eccentricities¹⁸. As a consequence, this relationship provides unambiguous information about the fronto-parallel characteristic of the surface with respect to the head. Unlike the VSR, the value of the HSR depends on the angle of slant of the surface about a vertical axis, as well as on e and D . The VSR (the size ratio of a vertical element), cannot be affected by the slant of the surface about a vertical axis and is dependent on e and D alone. O. J. Braddick (personal communication) has pointed out that unlike monocular perspective cues, the differential perspective cues described here could be exploited without the need to make assumptions about the homogeneity or isotropy of the elements making up the surface.

FIG. 3 In the experiments described here, observers sat in a modified Wheatstone stereoscope with mirrors at 45 deg to the line of sight and viewed the rear-projected images created on two translucent Mylar screens 57 cm from the observer's eyes. The images subtended 80° (horizontally) by 70° (vertically). The stereoscopic images were accurately synthesized to mimic the viewing of a flat, densely textured, fronto-parallel surface at a distance of either 28 cm or infinity. The simulated fronto-parallel surface surrounded a central rectangular region (25 by 20°) which contained horizontal sinusoidal corrugations of 0.2 cycles per degree and 10 arc min peak-to-trough amplitude specified by horizontal disparities. On separate trials, observers were asked to estimate either (1) the perceived peak-to-trough depth of the corrugations, or (2) the perceived periodicity (vertical separation) of the corrugations. Observers were allowed to choose their own units (inches or cm) to make the judgements. Because individual observers use the numbers of cm or inches of perceived depth in very different ways, the average amount of perceived depth was normalized for each observer using the average estimate of the amount of depth seen in the corrugations when the vertical perspective cues were correct for the 57-cm viewing distance (dashed line). The normalized perceived depth, averaged over 10 observers, together with the standard errors of the estimates, are plotted as a function of the simulated viewing distance (28 cm or infinity) for the three different conditions: (1) both vertical and horizontal perspective cues present (V + H), (2) only vertical perspective cues present (V) and (3) only horizontal perspective cues present (H). In all three cases the difference in perceived depth for the two simulated viewing conditions was statistically significant ($P < 0.05$). The 1.9:1 difference in the amount of perceived depth for the two simulated viewing distances when both vertical and horizontal perspective cues were present is very much less than would be required for complete depth constancy. The most likely reason for the incompleteness of the depth scaling is the presence of contradictory vergence angle information, which remained constant and appropriate for the 57-cm distance of the display screens throughout the experiments. The results also show that most of the effect on the perceived depth can be attributed to the presence of the vertical perspective cues although the small effect of horizontal perspective



cues was present in the estimates of eight out of ten observers and was statistically significant. Masking the displays down so that only the central 25 × 20° area was visible abolished the effect of the differential perspective manipulations. This result supports our speculation that the failure of Cumming *et al.*¹⁰ and Sobel and Collett¹¹ to find an effect of vertical disparities was due to the relatively small sizes of their displays. In contrast, the right-hand pair of bars show the average amount of perceived depth for the two simulated viewing distances in the 'window' condition in which the vertical and horizontal perspective cues were present only in the peripheral fronto-parallel regions and the central region containing the stereoscopic corrugations provided consistent perspective cues for the actual 57-cm viewing distance.

perspective cues appropriate to viewing at 28 cm. The average ratio of perceived depth in the two conditions over the 10 observers tested was nearly 1.9:1 (Fig. 3). When the corrugated surfaces contained only differential vertical perspective cues (vertical disparities) appropriate to infinity and 28-cm viewing, the average ratio of perceived depths in the two conditions was 1.7:1. In contrast, when the corrugated surfaces contained only differential horizontal perspective cues, the average ratio of perceived depth was only 1.15:1. In the same experiment, observers were also asked to estimate the perceived separation (size) between the peaks of the corrugations. These results showed a similar pattern, with observers reporting a larger separation between the peaks of the corrugations when the perspective gradients were appropriate to viewing the surface at infinity. The differences in perceived separation were smaller than those for perceived depth, which is consistent with the different scaling required for size and depth constancy^{1,4}.

Our results show that horizontal gradients of VSR between the two eyes, differential vertical perspective, do affect the amount of perceived depth in stereoscopic surfaces, as predicted by Mayhew and Longuet-Higgins' computational theory. In addition, the results show that gradients of HSR between the two eyes, differential horizontal perspective, which are similarly created by binocular viewing of a close, fronto-parallel surface, also affect the amount of perceived depth in stereoscopic surfaces, albeit to a smaller extent. Why should differential vertical

perspective cues be more effective than differential horizontal perspective cues in scaling depth and size? A plausible answer is that the VSR is a function of e and D alone, whereas the HSR is a function of e , D and the angle of slant of the surface about a vertical axis. Hence vertical perspective cues provide the only consistent and reliable information about the distance to the surface and these were found to be more effective in practice for depth and size scaling. Moreover, our results show that the amount of perceived depth and the perceived separation of corrugations (size) were both affected when the differential perspective cues were present only in the surrounding flat regions of the stereo images and were absent in the corrugated surfaces themselves (Fig. 3, far right). This result suggests that the human visual system exploits the fact that the differential perspective effects increase with increasing eccentricity and consequently pools this information¹⁶ across quite large visual extents to obtain an estimate of absolute distance which is used for scaling disparities. Finally, our most recent results^{17,18} have shown that (1) the differential perspective cues described here also affect the perceived absolute distance to the surface (such that a surface containing perspective cues appropriate for near viewing is perceived to be closer to the observer), and (2) the shape of the apparent fronto-parallel surface systematically varies with the differential vertical perspective cues generated by surfaces at different absolute distances from the observer, as Helmholtz³ informally demonstrated over a hundred years ago. □

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Visible flicker from invisible patterns

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USING a laser interferometer we can create grating patterns of high optical contrast (interference fringes) directly on the retina¹⁻³. With coarse fringe patterns, the alternating light and dark bars of the pattern can be seen, but the bars of the finest fringes are not subjectively resolved. We report here that when we rapidly modulate the contrast of a fine fringe pattern (keeping overall luminance constant), observers experience flicker, even if the fringes are too finely spaced to be perceived as a grating. For this flicker to be seen, the pattern needs to be resolvable by the photoreceptors themselves, but not necessarily by later stages of visual processing. It can be explained if, in man, signals associated with individual cone receptors do not depend linearly on light intensity, but instead are scaled by a fast sensitivity-regulating or light-adaptation mechanism. Contrast-modulation flicker is not demonstrable in rod vision; rod vision therefore lacks such a local adaptation process.

Subjects in our experiments viewed an interference fringe of time-varying contrast (Fig. 1, top panels). Generally, the spatial frequency of the fringe was so high that the stripes could not be resolved by the subject and the field looked uniform (except for laser speckle). The contrast fluctuated sinusoidally between

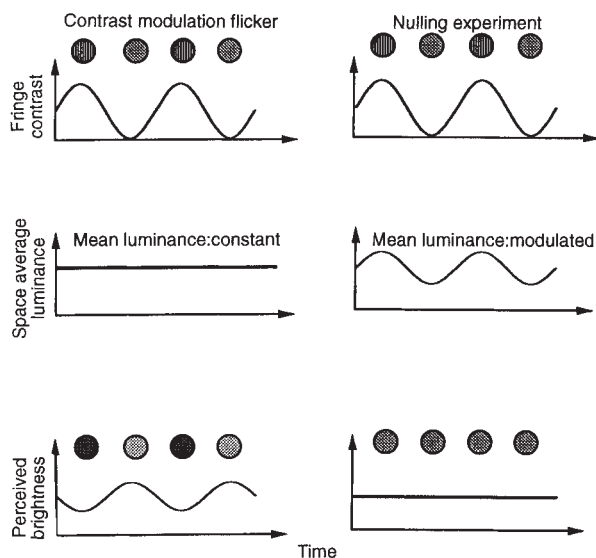


FIG. 1 The stimulus used to generate contrast-modulation flicker (left panel), and the stimulus used to null or cancel it (right panel). Fringe contrast (top left) varied sinusoidally between a peak value (generally unity) and zero. If luminance was constant during the modulation of contrast (middle left), flicker could be seen. The observer then adjusted the amplitude and phase of the nulling luminance modulation (middle right) in order to minimize the perceived flicker. It proved helpful to superimpose on the depicted stimulus a fixed 'pedestal' luminance modulation. In alternating 1-s intervals, distinguished by computer-generated beeps, this pedestal was arranged to alternately cancel or reinforce the contrast-modulation flicker. Any failure to null the contrast-modulation flicker revealed itself as a difference in the resultant flicker amplitude between these two intervals. The test field was a disc subtending 50' of visual angle, illuminated with 2,000 trolands (td) of red (633 nm) light from a He-Ne laser and enclosed in a uniform equiluminous surround. It was centrally fixated, and so fell within the central fovea where the photoreceptors are small enough to preserve high optical contrast for fringes as fine as 100 cycles per degree, far above the resolution limit².

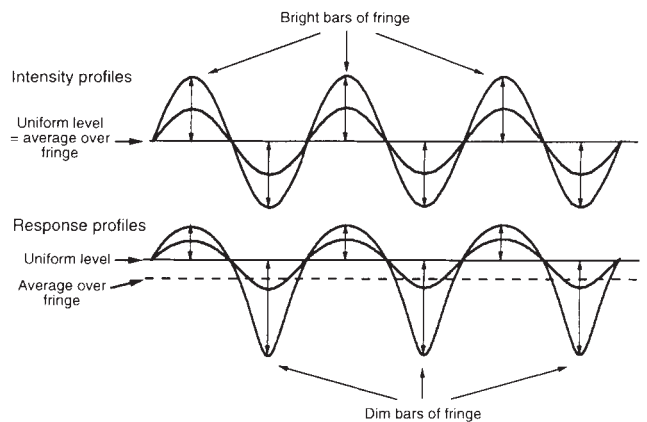


FIG. 2 Top, each curve is a cross-section of the light intensity profile over the test field, at some moment during the modulation of fringe contrast. Horizontal line is when fringe contrast is zero (uniform field); other curves show fringe at intermediate or at peak contrast. Bottom, corresponding response profiles if the relation between response and intensity is compressively nonlinear. The average response with the fringe at peak contrast falls below the response to the uniform field.

a peak value (generally unity) and zero. Our subjects saw flicker, as if the overall luminance of the field was changing, even though the total amount of light in the test field remained constant during the modulation (Fig. 1, lower left panels). This contrast-modulation flicker arises not simply from detection of the local changes of intensity that occur within the test field, but from a reduction in the perceived overall brightness of the field when the contrast is increased: we could cancel, or null the flicker by modulating the overall physical luminance of the field in synchrony with the modulation of contrast (Fig. 1, lower right). At a suitably adjusted amplitude of this nulling luminance modulation, the perceived flicker was minimized and the field appeared almost steady as well as uniform.

Contrast-modulation flicker can be explained if each cone photoreceptor's response depends in a nonlinear way on light intensity⁴, and if later neurons sum these nonlinear signals over their receptive fields to determine the perceived overall luminance of the unresolved fringe. When the interference pattern, having reached peak contrast, is then succeeded by the physically uniform phase of the test field, the luminance of the light bars goes down by as much as the luminance of the dark bars goes up. This keeps the overall space-average luminance constant (Fig. 2, upper curves) and, if each photoreceptor's signal were a fixed linear function of local intensity, the average of the receptor signals would remain constant as well. But if each cone receptor's response is a compressively nonlinear function of intensity (or equivalently, if individual cone signals are adaptively scaled by a sensitivity-regulating process), then the response profiles will be asymmetrically distorted as shown by the lower curves in Fig. 2, and the decrement in response in the previously bright bars will be less than the increment in the previously dark bars. So the space-averaged response will be greater during the uniform phase (Fig. 2, continuous horizontal line) than it was for the fringe (dashed line).

In our nulling experiment, this difference is compensated by a visually equivalent increase in overall physical luminance when the fringe is near peak contrast. The nulling luminance variation was more than a factor of two between maximum and minimum under some conditions, but it depended strongly on fringe peak contrast: halving the fringe contrast reduced the equivalent luminance excursion about fourfold. This roughly quadratic dependence on fringe contrast is theoretically expected if the cone output is a smoothly compressive nonlinear function of intensity, with a large quadratic term in its power series expansion.

The responsible nonlinear process must be at a stage within the visual system where the fringe can be resolved. If it takes place within the photoreceptor cells, contrast-modulation flicker should require only that the fringe be present with high contrast at the photoreceptor level. Whether it is resolved perceptually, or by later neural stages of the visual system, would then be irrelevant: the flicker amplitude should depend entirely on the fringe contrast as seen by the cones themselves, which is limited mainly by the size of the individual cone receptors. Figure 3 supports this prediction. The finer the fringe, the less the flicker (Fig. 3a); but the equivalent amplitude decreases only very slowly as the fringe spatial frequency increases past the visual resolution limit (here just over 60 cycles per degree of visual angle). Contrast-modulation flicker is measurable even above 100 cycles per degree. Evidently the responsible nonlinear elements resolve optical detail much better than does the entire visual system of the human observer.

Figure 3b shows just how little spatial integration of light intensity at the input to the nonlinear elements is implied by the results of Fig. 3a. The gaussian curve is the point spread function corresponding to the curve of Fig. 3a. Evidently the nonlinearity is preceded by spatial integration of light intensity over a region spanning only 18 seconds of arc, or $1.5 \mu\text{m}$ on the retina (full width at half height). This is equal to the smallest histological estimate of the diameter of the light-collecting inner segment of the central foveal cones^{5,6}, and provides a functional estimate of the cone's light-collecting area that is in rough agreement with other recent evidence⁷. This leaves no margin for any neural spatial integration. The nonlinear process is therefore local and early: either it is inside the cones themselves, or otherwise it operates independently on the signals from each cone, before further spatial integration obliterates the representation of the finest fringes at later neural stages and in perception.

We have investigated the dynamic aspect of this early visual nonlinearity by varying the modulation frequency (the frequency at which the fringe appears and disappears). The equivalent (nulling) amplitude remains substantial up to 30 Hz (Fig. 4,

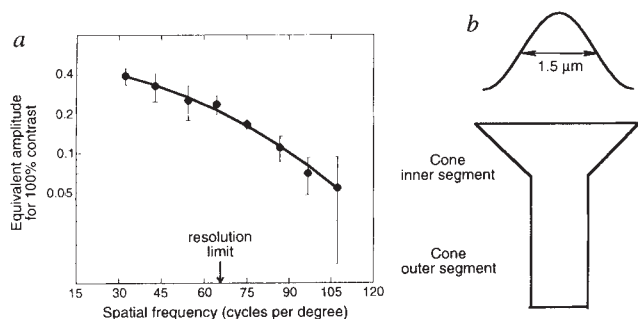


FIG. 3 a, Filled circles, contrast-modulation flicker as a function of fringe spatial frequency for test fields fluctuating sinusoidally at 15 Hz between unity contrast and zero contrast. The equivalent (nulling) luminance modulation approaches 40% (a sinusoidal modulation of overall luminance between 1,200 and 2,800 trolands) at low fringe frequencies, and decreases only slowly with increasing frequency, remaining measurable at 100 cycles per degree of visual angle. Error bars are the 95% confidence intervals, based on variation among the mean settings from four different sessions. Curve is best-fitting gaussian. Two other subjects (D.I.A.M. and one other subject naive as to the purpose of the experiment) were tested; they yielded similar results. The indicated visual resolution limit is the highest fringe frequency at which a two-alternative forced-choice discrimination between horizontal and vertical fringes of unity contrast could be made with 75% reliability. b, point spread function for light intensity at the input to the nonlinear element, compared with the dimensions of a central foveal cone photoreceptor⁶. Effective contrast at the input to the nonlinear elements was assumed proportional to the square root of the equivalent amplitude from a; the corresponding radially symmetrical point spread function shown here is gaussian in spatial coordinates, and is given by the Fourier transform of the function relating contrast to spatial frequency.

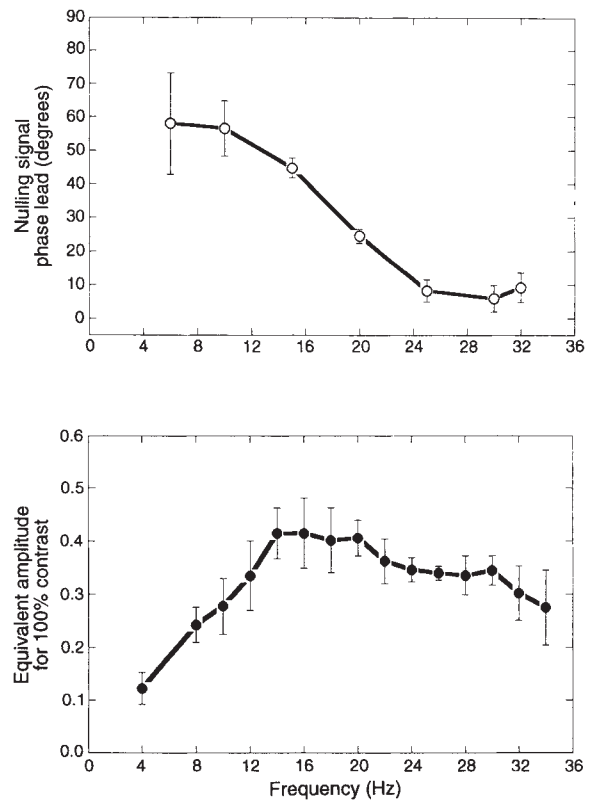


FIG. 4 Open circles, phase of luminance modulation optimally effective in perceptually cancelling contrast-modulation flicker, for a range of temporal frequencies. Spatial frequency 30 cycles per degree. Positive phase values mean the luminance peak had to precede the contrast peak. Error bars are the 95% confidence intervals, based on variation among the mean settings from four different sessions. Results for the two other subjects were similar. Filled circles, amplitude of luminance modulation required to cancel contrast-modulation flicker at different modulation frequencies.

filled circles). At the lower frequencies, the luminance modulation for an optimal null at high peak contrast has to be considerably advanced in phase relative to the modulation of contrast (empty circles, Fig. 4). This excludes instantaneous compression (with a fixed relationship between input intensity and output, as assumed in Fig. 2) as a model for the nonlinear process, because that model predicts no phase mismatch⁴; it also excludes models in which time-average light exposure speeds the visual response as well as reducing its sensitivity^{7,8}, because these predict a nulling signal lag, not a lead⁹. But the phase lead is expected if a relatively fast sensitivity-regulating process scales sensitivity roughly in inverse proportion to the recent input level for each cone. Its disappearance at 25–30 Hz is expected if the adaptation process fails to follow the input closely at these high frequencies⁹.

Our results suggest that a major component of human photopic (cone) visual adaptation to light is very fast and strictly local. A strictly local process could reside either within the cones themselves or in later neural structures (such as the cone-bipolar synapse) that are fed by single cones. We find (in preparation) that contrast-modulation flicker is visible down to low photopic light levels, below 1,000 quanta per cone per second. This intensity range is comparable with the range of adaptation in monkey gross receptor potentials (isolated with aspartate in the monkey foveal electroretinogram⁴), but extends well below the range reported for cone outer segment photocurrent¹⁰. The situation for rod vision is quite different: contrast-modulation flicker in the rod system (measured 15 degrees extrafoveally with 515 nm laser light on a deep red steady background) is absent or minimal. This supports other evidence^{2,11–14} that in human

vision the cone system, but not the rod system, has substantial receptor-specific sensitivity regulation. □

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Proliferation of oligodendrocyte precursor cells depends on electrical activity in axons

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OLIGODENDROCYTES myelinate axons in the vertebrate central nervous system. It would, therefore, make sense if axons played a part in controlling the number of oligodendrocytes that develop in a myelinated tract. Although oligodendrocytes themselves normally do not divide, the precursor cells that give rise to them do. Here we show that the proliferation of oligodendrocyte precursor cells in the developing rat optic nerve depends on electrical activity in neighbouring axons, and that this activity-dependence can be circumvented by experimentally increasing the concentration of platelet-derived growth factor, which is present in the optic nerve and stimulates these cells to proliferate in culture. These findings suggest that axonal electrical activity normally controls the production and/or release of the growth factors that are responsible for proliferation of oligodendrocyte precursor cells and thereby helps to control the number of oligodendrocytes that develop in the region.

To examine the role of axons in the proliferation of oligodendrocyte precursors, we transected one of the optic nerves in postnatal day 8 (P8) rats. After 4 days, the average number of mitotic figures per section was 90% lower in transected nerves than in the uncut control nerves (Fig. 1*a, b*). To test the possibility that the axons normally display a glial mitogen on their surface (as has been suggested for axons in the peripheral nervous system (PNS)¹), which is lost when the axons degenerate and are phagocytosed after transection, we studied the effect of

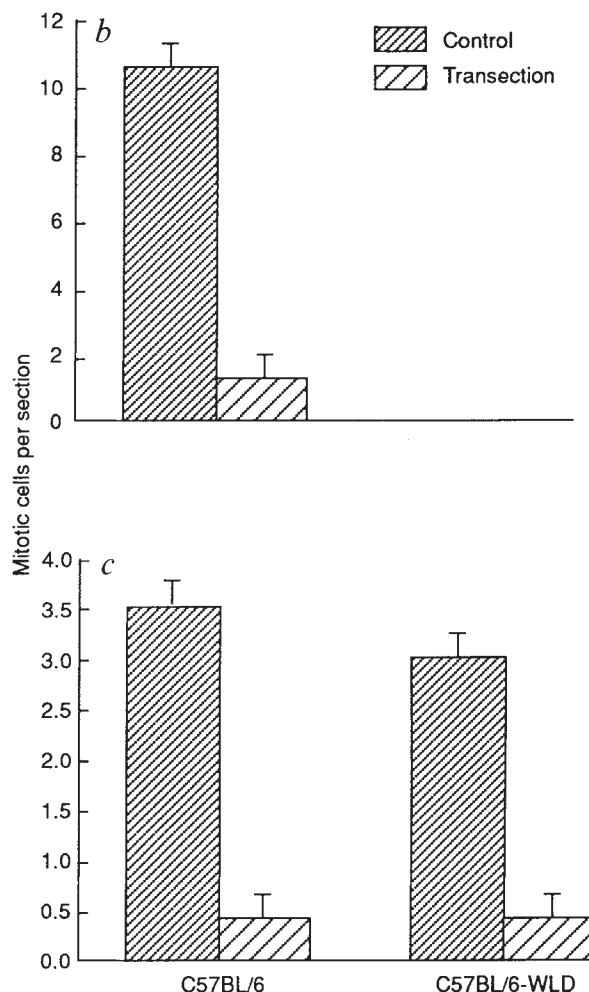
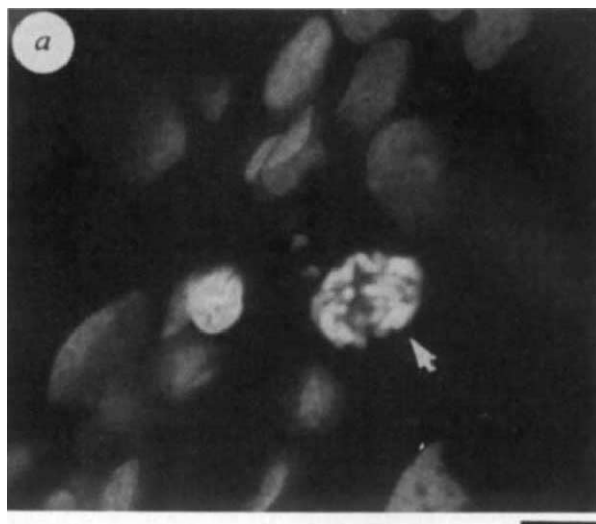


FIG. 1 The effect of nerve transection on cell proliferation in the developing optic nerve in rats (*b*) and in C57BL/6 and C57BL/6-WLD mice (*c*). A typical mitotic figure in a P12 optic nerve section stained with the nuclear counter-stain, propidium iodide, is shown in *a*.

METHODS. P8 (*a, b*) rats or P12 (*c*) mice were anaesthetized with ether and the right optic nerve was cut just behind the eyeball. Four days later, the rats were reanaesthetized with ether and perfused with 4% paraformaldehyde, as previously described². The optic nerves were incubated in 4% paraformaldehyde at 4 °C overnight, transferred to 30% sucrose in PBS until equilibrated, frozen in OCT compound (Miles) and cut into 8 μ m longitudinal sections, which extended the entire length of the nerve, with a Bright cryostat. The sections were collected onto gelatinized glass microscope slides, air dried, post-fixed in 70% ethanol for 10 min at -20 °C, and stained with propidium iodide (4 μ g ml⁻¹) in minimal Eagle's medium buffered with HEPES (MEM/HEPES) and containing DNase-free RNase A (100 μ g ml⁻¹) for 30 min at 37 °C. The slides were washed three times in PBS and mounted in Citifluor. The number of mitotic figures per section was determined by averaging the number of mitotic figures counted in five optic nerve sections per animal. In this and the following figures, the results obtained in four animals are shown as means $\pm$ s.e.m. The section shown in *a* was examined with a MRC-600 laser-scanning confocal imaging system in conjunction with a Nikon Optophot microscope. Scale bar, 8 μ m.

transection on mitosis in postnatal optic nerves of C57B1/6 and C57B1/6-WLD mice. C57B1/6-WLD mice have a genetic defect that prolongs the survival of the distal stumps of transected axons in both the PNS and central nervous system (CNS)^{2,3}. Despite the lack of axonal degeneration in the transected optic nerves of C57B1/6-WLD mice (which we confirmed by labelling with the RT97 monoclonal antineurofilament antibody⁴), the decrease in mitotic cells after transection was not significantly different in these mice than in normal C57B1/6 mice (Fig. 1c), making it unlikely that the loss of an axolemmal mitogen is the cause of the decrease in proliferation.

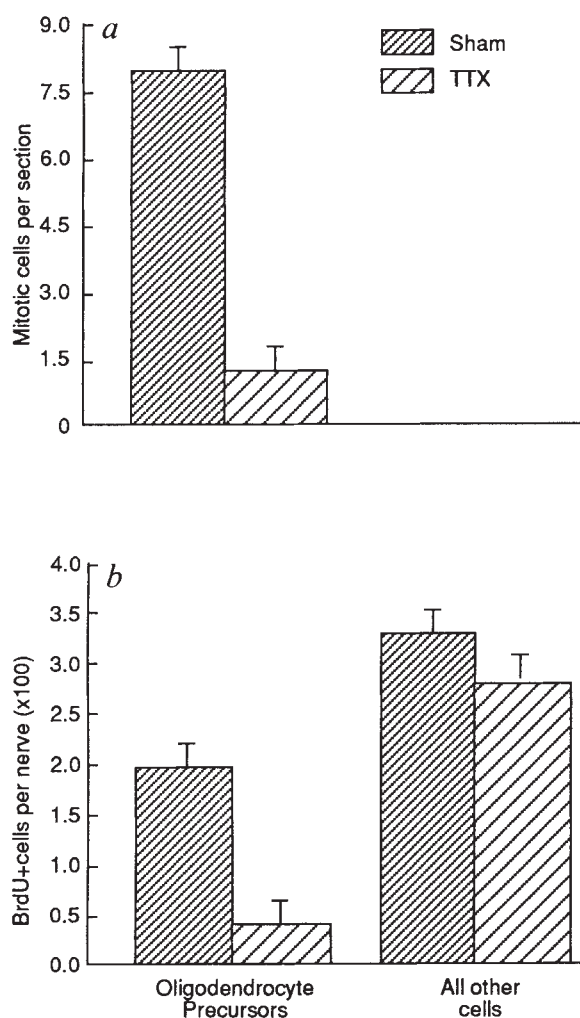


FIG. 2 The effect of an intraocular injection of tetrodotoxin (TTX) on mitosis (a) or BrdU incorporation (b) in the developing rat optic nerve.

METHODS. TTX ($0.5 \mu\text{l}$ TTX; 10^{-4} M) was injected slowly over about 2 min with a $5 \mu\text{l}$ Hamilton syringe through a 34 gauge needle into the right eye of ether-anaesthetized P15 (a) or P7 (b) rats as previously described⁵. Control animals received an identical volume of the corresponding acetate buffer vehicle (pH 7). Injections were made into the vitreous by placing the needle just posterior to the corneoscleral junction, and the success of the injection was confirmed by ensuring that the pupillary response was eliminated. The optic nerves were studied after 2 days. In a, optic nerve sections were prepared as described in Fig. 1. In b, cells in S phase were labelled *in vivo* by an intraperitoneal injection of BrdU (0.1 mg g^{-1} body weight; Boehringer-Mannheim), which is incorporated into replicating DNA²¹. The animals were sacrificed 60 min later and optic nerve cell suspensions were prepared as previously described⁷. Cells were plated onto poly-D-lysine (PDL)-coated coverslips and cultured for several hours before fixation with 4% paraformaldehyde for 90 s at room temperature. Cells were double-labelled by immunofluorescence with monoclonal anti-BrdU²² and A2B5 antibodies²³ as previously described⁷. Oligodendrocyte precursor cells were identified by their characteristic morphology and by the presence of A2B5 (ref. 24).

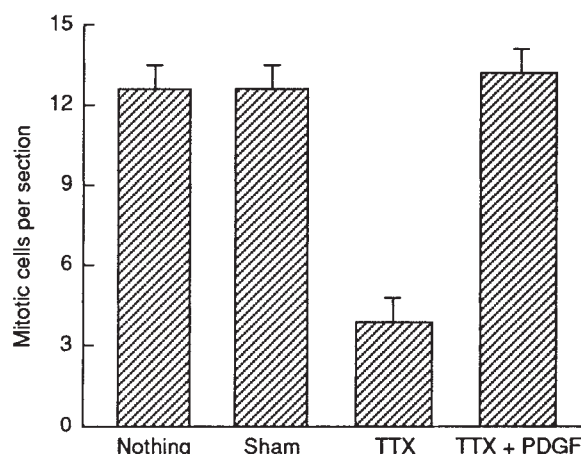


FIG. 3 The effect of increasing PDGF levels on cell proliferation in normal developing optic nerve and after TTX treatment.

METHODS. TTX was injected into the right eye of P10 rats and both optic nerves were examined 2 days later, as described in Fig. 1. Some animals also received a subarachnoid injection of COS-7 cells that were transiently transfected with a PDGF-A complementary DNA and which secreted PDGF into the cerebrospinal fluid⁷. The COS-7 cells were transfected 18 hours before injection with the PHYKA5 PDGF plasmid expression vector, as previously described⁷. Two million cells in $3 \mu\text{l}$ were slowly injected under ether anaesthesia through the right frontal skull into the subarachnoid space using a $10 \mu\text{l}$ Hamilton syringe. Identical injections of mock-transfected COS-7 cells had no effect on mitosis in postnatal optic nerves⁷.

To test the possibility that electrical activity mediates the axonal effects on glial cell proliferation, we injected tetrodotoxin (TTX) into one eye of P15 rats to eliminate the electrical activity of retinal ganglion cell axons⁵ and examined the nerves 2 days later. The number of mitotic cells per section was decreased by about 86% in TTX-treated rats compared to sham injected rats (Fig. 2a). To determine if the effect of TTX was specific to oligodendrocyte precursor cells, we injected TTX into one eye of P7 rats and 2 days later injected the rats with the thymidine analogue bromodeoxyuridine (BrdU) to label cells synthesizing DNA. One hour later we dissociated the nerves, cultured them for a few hours, and double-labelled them with an anti-BrdU antibody and an antibody that labels oligodendrocyte precursor cells. The average number of BrdU-labelled oligodendrocyte precursor cells fell by 80% in the TTX-treated nerve, but there was no significant change in the numbers of BrdU-labelled cells in the remaining population, indicating that TTX specifically blocks proliferation of the oligodendrocyte precursor cells (Fig. 2b). The effect is unlikely to be directly on the precursor cells, which also have TTX-sensitive Na^+ channels⁶, as TTX did not inhibit the platelet-derived growth factor (PDGF)-stimulated division of purified oligodendrocyte precursor cells⁷ *in vitro* (Table 1).

To determine whether TTX blocks proliferation of oligodendrocyte precursor cells by decreasing the production or release of a mitogen, we injected TTX into one eye of P10 animals and examined their optic nerves 2 days later; in these experiments, some of the animals also received a subarachnoid injection of COS cells that were genetically engineered to secrete the AA form of PDGF into the cerebrospinal fluid, from where it is delivered to the optic nerve⁷. The animals that received the PDGF-secreting COS cells were protected from the anti-proliferative effects of nerve transection (Fig. 3), whereas animals that received nontransfected COS cells were not (data not shown). These results, together with previous observations that isolated oligodendrocyte precursor cells in the absence of neurons retain high sensitivity to the mitogenic effects of PDGF⁷, suggest that electrical activity in axons normally stimulates the

production and/or release of mitogens that specifically stimulate the proliferation of oligodendrocyte precursor cells.

The recent discovery that many CNS neurons make PDGF^{8,9}, raises the possibility that electrically active axons release PDGF, which stimulates oligodendrocyte precursors to proliferate. Axons, however, have not been shown to secrete protein signalling molecules, whereas type-1 astrocytes, a major class of glial cells in the optic nerve, have; moreover, type-1 astrocytes make the AA form of PDGF both *in vivo* and *in vitro*^{10,11}. It seems likely that type-1 astrocytes are a major source of PDGF in the developing rat optic nerve, as the number of oligodendrocyte precursor cells in the nerve appears to be limited by the amount of PDGF present, and the number of these precursor cells plateaus at P14, just when the number of type-1 astrocytes plateaus⁷. Thus we favour the possibility that electrically active axons produce a signal that stimulates type-1 astrocytes to produce and/or release PDGF. Consistent with this possibility, we find that optic nerve type-1 astrocytes in serum- and neuron-free cultures do not secrete molecules that stimulate oligodendrocyte precursor cells to proliferate *in vitro* (our unpublished observations). An alternative possibility is that electrically active axons release substances such as K⁺ or glutamate that potentiate the mitogenic effect of PDGF or other growth factors¹²⁻¹⁴. As shown in Table 1, however, we have failed to find evidence that K⁺ or glutamate potentiate the mitogenic effect of PDGF on purified oligodendrocyte precursor cells.

Whatever the mechanism, our findings suggest that axonal electrical activity normally stimulates the proliferation of oligodendrocyte precursor cells, thereby increasing the number of oligodendrocytes that develop locally. These findings may account for the previous reports that premature eye opening accelerates optic nerve myelination¹⁵, whereas dark rearing delays it¹⁶. They may also explain the observations that eye removal profoundly inhibits gliogenesis in the optic tectum of the frog¹⁷ and mouse¹⁸. Electrical activity has previously been found to affect glial development: intracranial infusion of TTX into embryonic cats altered the ultrastructure of glial cells in the optic nerve¹⁹. Our results also raise the possibility that demyelinating lesions that cause a conduction block along axons, as occurs in multiple sclerosis²⁰, may fail to remyelinate in part because the silent axons fail to stimulate oligodendrocyte precursor cells to proliferate.

This study raises several important questions. How do electrically active axons signal oligodendrocyte precursor cells to

divide? Do active axons stimulate developing Schwann cells or their precursors to divide? Do similar mechanisms operate to mediate communication between active neurons and neighbouring glial cells in the adult nervous system? The answers should have important implications for understanding how the normal nervous system develops and functions and how it responds to injury and disease. □

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Secretion of β -amyloid precursor protein cleaved at the amino terminus of the β -amyloid peptide

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THE accumulation in brain of senile plaques containing β -amyloid protein (A β) is a defining feature of Alzheimer's disease¹⁻³. The amyloid precursor protein (APP)⁴ from which A β is derived is subject to several genetic mutations which segregate with rare familial forms of the disease, resulting in early onset of dementia and plaque formation⁵⁻⁹, suggesting that APP metabolism plays a causal role in the disease. Various cell types have been shown to release a soluble form of A β , thus allowing for the *in vitro* study of A β generation¹⁰⁻¹². We report here evidence that a substantial portion of the APP secreted by human mixed brain cell cultures, as well as that present in cerebrospinal fluid, is of a novel form cleaved precisely at the amino terminus of A β , suggesting that a secretory pathway is involved in A β genesis.

The APP is thought to be cleaved at a single non-amyloidogenic site within the A β sequence before secretion¹³⁻¹⁵. The demonstration of secreted forms of APP cleaved at a site other than that previously described is confounded by the fact that the secreted portion of APP is a glycoprotein translated from

TABLE 1 The effect of TTX, K⁺ and glutamate on BrdU incorporation by PDGF-treated oligodendrocyte precursor cells

Factors added	BrdU ⁺ cells (%)
None	0
PDGF	13.3 ± 0.4
PDGF (10 ng ml ⁻¹)	58.2 ± 2.3
PDGF + TTX (10 μ M)	12.9 ± 0.8
PDGF + glutamate (1 μ M)	11.1 ± 1.1
PDGF + glutamate (10 μ M)	3.1 ± 0.6
PDGF + glutamate (100 μ M)	0
PDGF + K ⁺ (10 mM)	13.4 ± 0.6
PDGF + K ⁺ (25 mM)	13.3 ± 0.5

About 10,000 oligodendrocyte precursor cells, purified to greater than 99.9% homogeneity by sequential immunopanning as described previously⁷, were plated onto PDL-coated wells of a 96-well Falcon culture dish in 100 μ l of serum-free Dulbecco's modified Eagle's medium containing insulin (5 μ g ml⁻¹), transferrin (100 μ g ml⁻¹), bovine serum albumin (100 μ g ml⁻¹), progesterone (60 ng ml⁻¹), putrescine (16 μ g ml⁻¹), sodium selenite (40 ng ml⁻¹), thyroxine (40 ng ml⁻¹), triiodothyronine (30 ng ml⁻¹) and PDGF-AA (Peprotech; 0.5 ng ml⁻¹, unless otherwise noted), TTX, K⁺ or glutamate. After 24 h, BrdU (10 μ M) was added, and 24 h later, the cells were fixed and labelled with an anti-BrdU monoclonal antibody²². The results shown are means $\pm$ s.e.m. of three cultures. The decreasing BrdU incorporation with increasing glutamate concentration was due to cell death.

several alternatively spliced isoforms^{16–18} that migrate in SDS-polyacrylamide gel electrophoresis (PAGE) as a fairly broad set of bands of apparent M_r s between 95–125K (ref. 19). Golde *et al.* constructed a series of APP deletion mutants whose processing was examined in 293 kidney cells. By this method small changes in the molecular mass of secreted APP would be detected if alternative cleavage sites were used. Under these conditions, only a single secretase cleavage site within the A β region was detected²⁰.

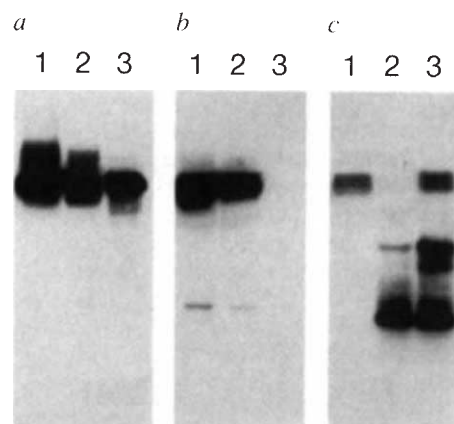
We used a different approach that employed human brain cultures and cerebrospinal fluid (CSF), sources that contain relatively high levels of soluble A β , as detected by enzyme-linked immunosorbent assay (ELISA)¹⁰, to search for alternative processing of APP in systems more analogous to human brain. The strategy was to use a resin-bound monoclonal antibody (antibody 6C6; ref. 21 and Fig. 1), which recognizes the N-terminal fragment of A β remaining on the previously described secreted APP (A β residues 1–15), to immunodeplete various samples of this previously identified form. As can be seen (Fig. 1, lane C2), this resin effectively removes the secreted APP containing A β 1–15. However, substantial APP immunoreactivity produced by human mixed brain cells is not captured by the resin (lane A2). To characterize this novel secreted form of APP lacking an A β epitope, we raised an antibody against APP residues 591–596 (numbering is as for the 695 form⁴). This antibody, termed 92, recognized the species of APP not captured by the resin, (lane B2) but did not react with the secreted form of APP containing A β 1–15 (lane B3). Competition studies with synthetic peptides

suggest that a free carboxy-terminal methionine is required for reactivity with antibody 92, as peptides one amino acid longer or shorter do not compete effectively (Fig. 2). The same pattern of peptide competition was observed in the cell culture supernatants (data not shown), supporting the contention that Met 596 ending APP is present both *in vitro* and *in vivo*.

A series of pulse-chase experiments revealed that antibody-92-immunoprecipitable material is also produced by 293 cells over-expressing either the 695 or 751 forms of APP (Fig. 3, lanes 5 and 8), although the fraction of APP that is truncated is considerably less than that seen in the mixed brain cell cultures. Both in transfected 293 cells and in human mixed brain cell cultures, antibody-92-immunoprecipitable material can be resolved from 6C6 reactive APP by low percentage (5%) SDS-PAGE (lanes 4, 5, 7, 8, 10 and 11). Note that mixed brain cell cultures secrete forms of APP of lower apparent molecular mass (lanes 10 and 11, arrows), both 695 and Kunitz protease inhibitory domain (KPI)-containing, in addition to the forms comigrating with secreted APP species in 293 cells. As these lower- M_r forms are reactive with antibodies 92 and 6C6, their increased gel mobility must be due to post-translational modification at other than the C termini. In the mixed brain cell cultures, the alternative processing of KPI-containing APP forms to produce antibody 92-reactive material is less apparent, although faint comigrating bands are observed with antibody 92 and anti-KPI immunoprecipitations (lanes 11 and 12)²¹. Kennedy *et al.*²² reported that CSF contains two major APP species lacking the partial A β sequence and which appear to be derived solely from

FIG. 1 Demonstration of truncated APP in conditioned medium from human mixed brain cell cultures. Sample 1 is the conditioned-medium from culture, sample 2 is the medium depleted of 6C6-reactive APP and sample 3 is the material extracted from the 6C6 resin. *a*, Probed with anti-5 (ref. 24), a polyclonal antibody which was raised against the APP sequence 444–592. *b*, Probed with antibody 92. *c*, The result of probing with 10D5, a monoclonal antibody which recognizes A β 1–16 (ref. 21). The uppermost band in each panel has an apparent M_r of $\sim 100K$ (based on molecular mass markers, data not shown). The lower M_r bands observed in C2 and C3 and not seen in C1 are derived from immunoglobulin subunits extracted from the 6C6 resin and are recognized by goat-anti-mouse alkaline phosphatase conjugate independent of a primary antibody (data not shown).

METHODS. The monoclonal 6C6 was raised and screened in the same manner as 10D5 (ref. 21) using a synthetic peptide containing A β residues 1–28 (coupled to rabbit serum albumin) as the immunogen. Both 10D5 and 6C6 recognize an epitope within the first 16 amino acids of the A β sequence. Preliminary experiments suggested 6C6 was more efficient in immunoprecipitation and thus was used as the capture antibody. To prepare the 6C6 resin, 4 ml affi-gel 10 (Bio-Rad) was washed with cold water and combined with 3 ml 6C6 (12.5 mg ml^{-1} in PBS, 0.5 M NaCl). The coupling proceeded overnight at 4 °C with gentle shaking. Tris (1 M, 400 μl), pH 8.0 were then added and the shaking continued for 40 min. The resin was then washed with Tris-buffered saline, 0.05% Tween-20 (TTBS) exhaustively before use. The antibody 7H5 is described in ref. 21. The peptide *N*-acetyl-CISEVKM (single-letter amino-acid code) was conjugated to rabbit serum albumin which had been activated with sulphy-maleimido benzoyl-*N*-hydroxy-succinimide ester. The conjugate was sent to Josman Laboratories for production of antisera in rabbits by standard methodologies. During each inoculation, rabbits received 50 μg antigen in 0.1 ml injections subcutaneously at ~ 10 sites (500 μg per boost). The same peptide was coupled to sulphy-link gel (Pierce) for the affinity purification of the 92 antibodies from the IgG fraction. Fetal brain tissue was obtained through local agencies following all federal guidelines for fetal tissue research. Specimens were from 12–14-week-old fetal cadavers. Samples of cerebral cortex were rinsed twice with Hank's balanced saline solution (HBSS). Cortical tissue (2–3g) was placed in 10 ml cold HBSS to which 1 mg of DNase (Sigma D4513) was added. The triturated suspensions were filtered through Nitex nylon screens of 210 μm then 130 μm as described in ref. 25. Cells were collected by centrifugation and resuspended in neuronal medium (MEM supplemented with 10% fetal bovine serum, 1% glucose, 1 mM sodium pyruvate, 1 mM glutamine, 20 mM KCl). Polyethyleneimine-coated 100-mm dishes were seeded with 1.5×10^7 cells in 8 ml neuronal medium. The medium was exchanged twice weekly. All cultures in this study were grown at least 30 days *in vitro*. For serum-free growth conditions, neuronal medium was



replaced with defined medium (DMEM supplemented with 5 $\mu\text{g ml}^{-1}$ bovine insulin, 0.1 mg ml^{-1} human transferrin, 0.1 mg ml^{-1} BSA fraction V, $6.2 \times 10^{-8} \text{ g ml}^{-1}$ progesterone, $1.6 \times 10^{-5} \text{ g ml}^{-1}$ putrescine, $3.9 \times 10^{-8} \text{ g ml}^{-1}$ sodium selenite, $4.2 \times 10^{-8} \text{ g ml}^{-1}$ L-thyroxine and $3.3 \times 10^{-8} \text{ g ml}^{-1}$ tri-iodo-L-thyronine) and the supernatant was collected after 3 days. Cells were placed into defined medium for 3 days after which 10 ml medium were collected. EDTA (5 mM), leupeptin (10 $\mu\text{g ml}^{-1}$) and Tris-HCl (20 mM, pH 8.0) were added and the sample was spun at 30,000g for 20 min at 4 °C. To one half of the supernatant, 6C6 resin was added (200 μl of a resin with $\sim 5 \text{ mg ml}^{-1}$ 6C6 bound). Both samples were mixed gently for 6 h at 4 °C, the resin was pelleted and to the supernatant a second aliquot of resin was added. The samples were further mixed overnight at 4 °C. The combined resins were washed twice with TTBS, then briefly extracted twice with 1-ml aliquots of 0.1 M glycine, 0.1 M NaCl pH 2.8. The material extracted from the resin, the medium depleted by the resin and the starting medium were individually precipitated with 10% TCA at 0 °C for 1 h, the pellets were washed with acetone and then resuspended in 150 μl of SDS-PAGE sample buffer under reducing conditions and boiled. Twenty-five microliters of each sample were subjected to SDS-PAGE using 10–20% Tricrine gels (Novex). The proteins were transferred to ProBlot PVDF membranes overnight at 40V. Visualization of immunoreactive proteins used the TROPIX chemiluminescence system according to the manufacturer's directions for the AMPPD substrate. Primary antibody concentrations used were: anti-5, 0.1 $\mu\text{g ml}^{-1}$; 92, 2 $\mu\text{g ml}^{-1}$; 10D5, 2 $\mu\text{g ml}^{-1}$.

APP-695. Our results in mixed brain cell culture would support this interpretation and further predict these two species both end at Met596 (Fig. 3, lane 11). Together the data show that although both APP695 and APP751 are, to a minor extent, cleaved at the N terminus of A β in 293 cells, mixed brain cell cultures process a much larger fraction of APP695 by this pathway.

Measurements by ELISA of the levels of secreted APP (all forms) and A β in human mixed brain cell culture conditioned media have shown a molar ratio of about 3:1 (data not shown). On the basis of near equality of antibody 92 and 6C6 reactive secreted forms produced during pulse-chase studies (Fig. 3), sufficient truncated APP is produced to account for the level of A β observed *in vitro*.

The detection of a secreted form of APP by an antibody that appears specific for an epitope ending in Met 596, the separation in SDS-PAGE of this lower- M_r secreted form from the previously described secreted APP, and the lack of immunological crossreactivity of the two secreted forms with antibodies 92 and 6C6 suggest specific cleavage of APP between residues Met 596 and Asp 597. The temporal coincidence of the appearance of antibody 92 and 6C6-precipitable APP argues against the likelihood of a second proteolytic event occurring post-secretion, particularly because longer chase times of up to 20 h do not measurably alter the ratio of the antibody 92 and 6C6 reactive species (data not shown). We suggest the term β -secretase site to describe the cleavage of APP between Met 596 and Asp 597 as distinct from the originally defined α -secretase site^{14,15}.

The relative scarcity of this alternative cleavage in 293 cells is certainly a consideration in explaining why this form was not observed initially¹⁴. The deletion mutants studied by Golde *et al.* lack a large section of the APP protein backbone, including several sites of post-translational modification²⁰. This alteration of the APP substrate could suppress the alternative pathway that we observe in the processing of the native APP. A longer secreted form of APP, first described in PC12 cells²³, is produced by 293 cells¹¹ and is also not detected in the deletion mutant studies, supporting this possibility.

These results suggest APP can be processed in a potentially amyloidogenic manner during the course of secretion. This activity is particularly prominent in brain cell culture, a system

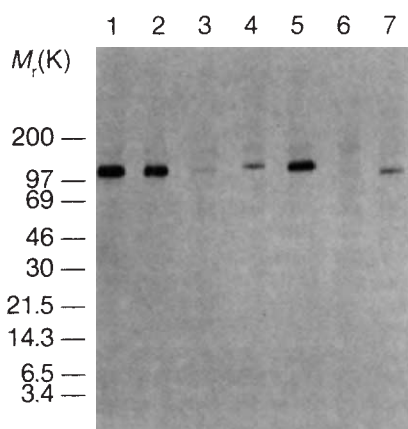


FIG. 2 Specificity of 92 antibodies. A human lumbar CSF specimen (1 ml) obtained from a 75-year old male was precipitated with 10% TCA to effect a 10-fold concentration and processed as described in Fig. 1, except that the gel well was a 4-cm slot. The 92 antibody was diluted to $6.7 \mu\text{g ml}^{-1}$ in 0.5 ml TTBS in the presence of various peptides, each at a concentration of $60 \mu\text{M}$, for 10 h at 4°C with gentle mixing. The antibody was then diluted eightfold in 1% gelatin/TTBS before incubation with immunoblot strips of CSF-derived material and processed as described in Fig. 1. The competing peptides are: lane 1, no competing peptide added; lane 2, GSGLTNIKTEEISEVK; lane 3, YSGLTNIKTEEISEVKM; lane 4, ISEVKM; lane 5, EISEVKMD; lane 6, CISEVKM; lane 7, YISEVKM.

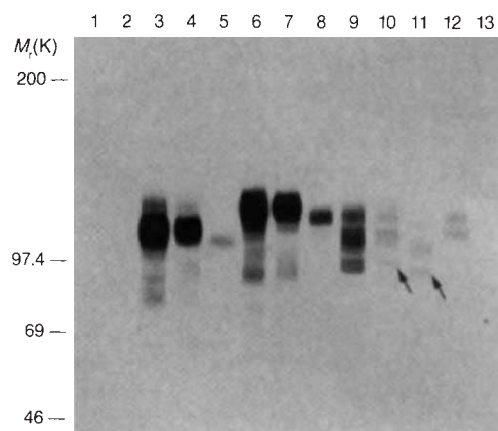


FIG. 3 Molecular mass heterogeneity of secreted forms of APP in immunoprecipitation detected by antibodies against different C termini in cell lines and primary human fetal brain cultures. Antibodies: anti-5 (against APP residues 444–592): lanes 3, 6 and 9; 6C6 (directed against A β peptide residues 1–16): lanes 4, 7 and 10; antibody 92 (against APP amino acids 591–596): lanes 5, 8 and 11; 7H5 (against APP-KPI) lane 12. Cells: lanes 1 and 3–5: 293 cells stably transfected with APP 695; lanes 2 and 6–8: 293 cells stably transfected with APP 751; lanes 9–13: human fetal brain cultures. Controls: lanes 1, 2 and 13: rabbit anti-mouse IgG antibody. Arrows: difference between low M_r secreted forms of APP 695 recognized by antibodies 6C6 and 92 (see text). SDS-PAGE was done on a 5% Laemmli gel. Human fetal brain cultures were established and maintained as described in Fig. 1. Cells used for this particular experiment were derived from gestational week 13. Cells were seeded at 1.5×10^7 cells per 10-cm dish and grown for 55 days in culture. The stably transfected 293 cells overexpressing APP have also been previously described¹⁹. Cells were grown in 10-cm dishes to subconfluency before use. Metabolic labelling and immunoprecipitation were done essentially as previously described^{24,26}. In brief, labelling was done in 10-cm dishes. Cells were washed in methionine-free medium, incubated for 20 min in 3 ml methionine-free medium supplemented with 0.5 mCi ^{35}S -methionine, washed in complete medium and chased for 2 h in 3 ml complete medium. Conditioned medium was collected and cleared at $3,000g$ for 10 min followed by preabsorption with Protein A-Sepharose (Pharmacia). Immunoprecipitation was done with 1.5 mg Protein A-Sepharose per sample. Antibody anti-5 was used at $2 \mu\text{g}$ per sample; 6C6, 7H5 and 92 were used at $10 \mu\text{g}$ per sample. Rabbit anti-mouse IgG ($5 \mu\text{g}$) was used with 6C6 and 7H5 as well as in the control samples. Precipitates were washed four times in TBS, 0.1% Nonidet P40, 5 mM EDTA, 1 mM PMSF, $10 \mu\text{g ml}^{-1}$ leupeptin.

which also releases considerable amounts of A β (ref. 10), and thus may be related to the vulnerability of brain tissue to formation of β -amyloid-containing plaque. Whether or not this alternative secretory pathway is linked to A β generation will require pharmacological reagents which selectively disrupt this activity. Determining whether the mutation in the region just preceding the N terminal of A β that is linked to a familial form of Alzheimer's disease⁹ influences the activity of this pathway will also be an important point of inquiry. If β -secretase activity is indeed an amyloidogenic event, then potential therapeutic opportunities exist for selective strategies to prevent the formation of A β which might not require suppression of lysosomal function¹². □

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Potassium channel stimulation by natriuretic peptides through cGMP-dependent dephosphorylation

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NATRIURETIC peptides inhibit the release and action of many hormones through cyclic guanosine monophosphate (cGMP)^{1,2}, but the mechanism of cGMP action is unclear³. In frog ventricular muscle and guinea-pig hippocampal neurons, cGMP inhibits voltage-activated Ca²⁺ currents by stimulating phosphodiesterase activity and reducing intracellular cyclic AMP^{4,5}; however, this mechanism is not involved in the action of cGMP on other channels⁶ or on Ca²⁺ channels in other cells^{7,8}. Natriuretic peptide receptors in the rat pituitary also stimulate guanylyl cyclase activity but inhibit secretion by increasing membrane conductance to potassium^{9,10}. In an electrophysiological study on rat pituitary tumour cells¹¹, we identified the large-conductance, calcium- and voltage-activated potassium channels (BK) as the primary target

of another inhibitory neuropeptide, somatostatin. Here we report that atrial natriuretic peptide also stimulates BK channel activity in GH₄C₁ cells through protein dephosphorylation. Unlike somatostatin, however, the effect of atrial natriuretic peptide on BK channel activity is preceded by a rapid and potent stimulation of cGMP production and requires cGMP-dependent protein kinase activity. Protein phosphatase activation by cGMP-dependent kinase could explain the inhibitory effects of natriuretic peptides on electrical excitability and the antagonism of cGMP and cAMP in many systems¹².

The effect of atrial natriuretic peptide (ANP) on voltage-activated currents from metabolically intact GH₄C₁ cells is illustrated in Fig. 1. ANP rapidly increased the steady-state outward current to 304 ± 60% of the control (*n* = 6). This effect was predominantly on BK channel activity because subsequent addition of 30 nM charybdotoxin reduced outward current below control levels (*n* = 2/2, data not shown). When outward current was blocked completely by replacing intracellular K⁺ ions with Cs⁺, ANP also decreased voltage-activated Ca²⁺ currents through dihydropyridine-sensitive channels (Fig. 1*d*), but this effect was much smaller, only 37 ± 15% (*n* = 5) at the peak of the current-voltage relation (Fig. 1*e*). The increase in outward current was unrelated to the decrease in Ca²⁺ current because the increase was also observed at voltages greater than +50 mV (Fig. 1*b*), where Ca²⁺ entry was negligible (Fig. 1*e*). Bath application of 1 mM 8-bromo-cGMP mimicked ANP action (Fig. 1*c, f*). In addition, 10 nM okadaic acid completely reversed the effect of both ANP and 8-bromo-cGMP on outward current (Fig. 1*b, c*), implicating protein phosphatase 2A in their action¹³. These electrophysiological effects appear identical to the effects of another inhibitory neuropeptide, somatostatin¹¹. Therefore, we investigated the effect of ANP and somatostatin on cGMP formation in GH₄C₁ cells.

FIG. 1 Atrial natriuretic peptide (ANP, 100 nM) and 8-bromo-cGMP (8br-cGMP, 1 mM) modulate voltage-activated currents in metabolically intact pituitary tumour cells. *a*, ANP increases voltage-activated outward current in physiological saline, an effect that is blocked by 10 nM okadaic acid (OA). *b, c*, Current-voltage relationship for the steady-state outward current in response to ANP or 8br-cGMP. *d*, ANP reduces inward current through voltage-activated, dihydropyridine-sensitive Ca²⁺ channels; okadaic acid was not tested. *e, f*, Current-voltage relationship for the peak inward current in response to ANP or 8br-cGMP. **METHODS.** GH₄C₁ cells were voltage-clamped through nystatin-perforated patches as described^{11,28}. The bath solution contained 140 mM NaCl, 5 mM KCl, 2 mM MgCl₂, 5 mM CaCl₂, 20 mM glucose, 1 μM tetrodotoxin, and 20 mM HEPES at pH 7.2. The pipette solution contained 115 mM KCH₃SO₃, 15 mM KCl, 5 mM MgCl₂ and 20 mM HEPES at pH 7.2. To isolate voltage-activated Ca²⁺ currents, K⁺ in the pipette was replaced by Cs⁺, and the concentration of Ca²⁺ in the bath was increased to 10 mM. ANP and 8-bromo-cGMP were diluted to their final concentration in the bath solution and perfused into the 0.5 ml bath at 2–3 ml min⁻¹. Experiments with ANP were done at 30–32 °C, whereas those with 8-bromo-cGMP were done at 19–23 °C.

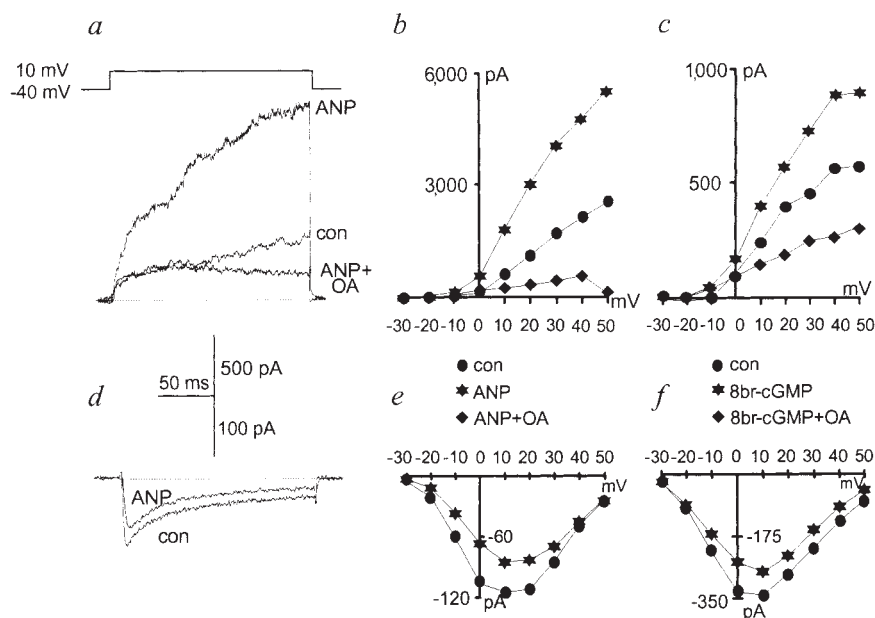


FIG. 2 Rat atrial natriuretic peptide (rANP) is a potent and specific stimulator of cGMP accumulation in GH_4C_1 cells. *a*, Time course of cGMP accumulation at 37 °C in the presence of 0.5 mM isobutylmethylxanthine (IBMX) under control conditions (○) or following addition of 100 nM rat ANP (●). *b*, Accumulation of cGMP after 30 min exposure to 100 nM ANP, 100 nM somatostatin (SRIF), or 50 μM sodium nitroprusside (NP). *c*, Concentration dependence of rANP effect on cGMP accumulation. *d*, Time course of cGMP accumulation (●) and outward current increase (○) in response to 100 nM rANP at 30 °C.

METHODS. GH_4C_1 cells were preincubated at 37 °C (*a-c*) or 30 °C (*d*) for 15 min in a standard salt solution: 118 mM NaCl, 4.6 mM KCl, 1 mM CaCl_2 , 10 mM glucose and 20 mM HEPES (pH 7.2). *a-c*, IBMX (0.5 mM) was added to inhibit phosphodiesterase activity. The cells were then exposed to the indicated agents in fresh solution with the same concentration of IBMX as the preincubation. The treatments were terminated after 30 min (or the time indicated). The solution was aspirated, and the cells were extracted at 4 °C for 60 min in 0.1 M hydrochloric acid. Cell extracts were acetylated and assayed for cGMP as described previously²⁹. Each point represents the mean $\pm$ s.e. of triplicate wells.

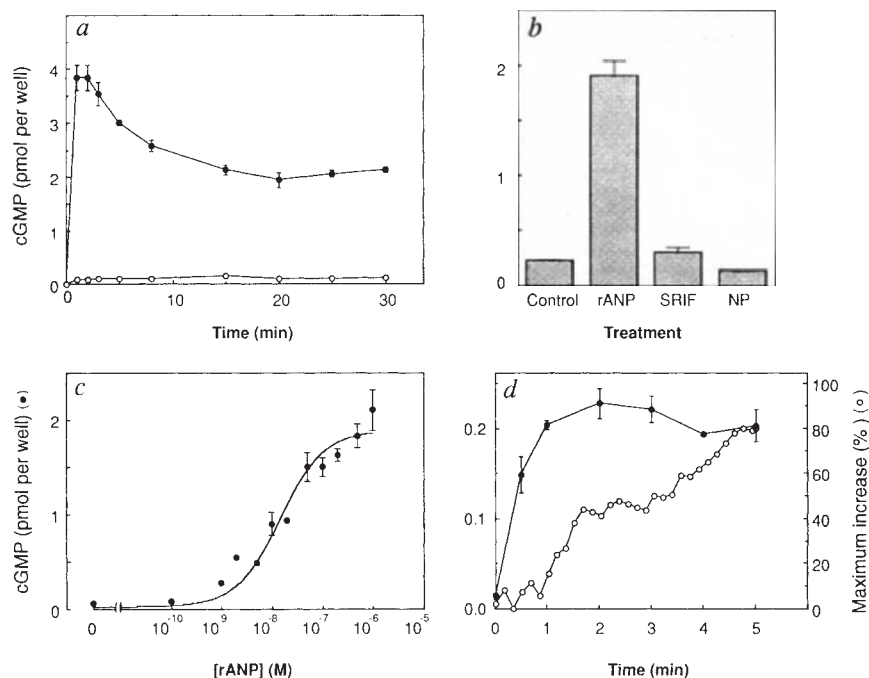
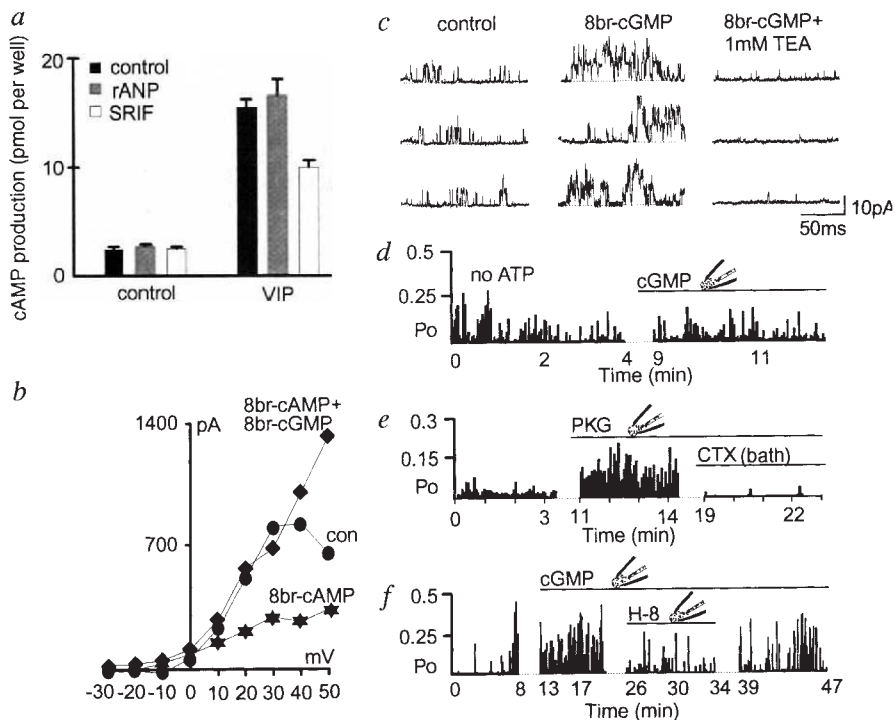


FIG. 3 Cyclic GMP activates a protein kinase. *a*, ANP has no effect on basal or VIP-stimulated cAMP accumulation. *b*, Bath application of 1 mM 8-bromo-cGMP to a metabolically intact cell stimulates steady-state outward current in the continued presence of 1 mM 8-bromo-cAMP, a hydrolysis-resistant derivative of cAMP. *c*, Bath application of 8-bromo-cGMP to a cell-free patch stimulates BK channel activity, which is blocked completely by 1 mM tetraethylammonium ions (TEA). *d*, 100 μM cGMP has no effect on BK channel activity in a cell-free patch in the absence of ATP. *e*, The catalytic fragment of the cGMP-dependent protein kinase (PKG) stimulates BK channel activity, which is blocked by charybdotoxin (CTX, 30 nM), in a cell-free patch. *f*, *N*-(2-(methylamino)ethyl)-5-isquinolinesulphonamide (H8, 600 nM)¹⁹ reversibly blocks the effect of cGMP on BK channel activity in a cell-free patch. **METHODS.** To assay cAMP levels, GH_4C_1 cells were preincubated at 37 °C for 60 min in the standard salt solution without IBMX. The cells were then exposed to 100 nM ANP or somatostatin (SRIF) in fresh solution. After 5 min, the solution was aspirated, and the cells were extracted with 0.1% trifluoroacetic acid in chilled 95% ethanol. Cell extracts were lyophilized, dissolved in 50 mM sodium acetate (pH 6.2), acetylated and assayed for cAMP as described previously¹³. Each point represents the mean $\pm$ s.e. of triplicate wells. To measure BK channel activity in cell-free membranes at 21 °C, outside-out patches were formed by omitting nystatin and adding 2 mM ATP-Mg and 1 mM BAPTA ($p\text{Ca} = 7$ or 8) to the solution in the patch pipette. After forming a gigohm seal, the patch was disrupted by suction and excised from the cell. The patch pipette was perfused by a modification of previously described methods^{4,30}. Channel activity was elicited by repeatedly (0.5 Hz) depolarizing the patch to +40 mV for 150 ms from a holding potential of 0 mV. The mean open probabilities for each depolarization are plotted as consecutive vertical lines. Bovine lung cGMP-dependent protein kinase was prepared as described previously³¹. To isolate the cGMP-independent 70 K fragment of the enzyme, 300 μg of the kinase



was incubated with 3 μg of trypsin (Sigma, Type 1) for 3 min at 30 °C. The reaction was terminated by adding 30 μg of soybean trypsin inhibitor and placing the mixture on ice. The catalytic fragment was isolated by gel filtration on a Sephadex G100 superfine column (0.7 $\times$ 9 cm) using Tris-buffered saline. The final preparation catalysed 24 pmol of phosphate incorporation to histone H2B per min per μl of enzyme, and was not dependent on cGMP for activity. A 1:30 dilution of this preparation was applied to the cytoplasmic side of outside-out patches.

In the presence of 500 μM isobutylmethylxanthine (IBMX) to inhibit cyclic nucleotide phosphodiesterases¹⁴, ANP rapidly stimulated cGMP formation (Fig. 2a). Cyclic GMP levels increased 40-fold at the peak of the response and remained elevated for more than 30 min with ANP present (Fig. 2a). Under the same conditions, somatostatin had no effect on cGMP production (Fig. 2b). Furthermore, overnight exposure to 100 ng ml⁻¹ pertussis toxin, which blocked the response to somatostatin¹¹, did not prevent ANP from increasing outward current ($n=3/3$, data not shown). Thus ANP and somatostatin activate different signalling pathways in rat pituitary tumour cells; somatostatin activates a pertussis-toxin-sensitive G protein¹⁵, whereas ANP activates a guanylyl cyclase. This guanylyl cyclase is probably intrinsic to the ANP receptor because 50 μM nitroprusside, which stimulates soluble guanylyl cyclases in other preparations³, had no effect on cGMP formation in GH₄C₁ cells (Fig. 2b) or on BK channel activity ($n=0/3$, data not shown). In contrast, ANP stimulated half-maximal cGMP formation at a concentration of 14.8 ± 4.5 nM (Fig. 2c). Without IBMX to inhibit phosphodiesterases, the absolute increase in cGMP was 10-fold lower (Fig. 2d). Nevertheless, when ANP was applied at the same concentration and temperature, cGMP formation always preceded the increase in outward current (Fig. 2d), consistent with a direct role for cGMP in ANP action.

Three classes of cGMP-binding proteins have been identified: phosphodiesterases, ion channels and protein kinases^{3,16}. Although cGMP alters phosphodiesterase activity in many systems^{4,5,17}, the effect of ANP on outward current in GH₄C₁ cells did not result from changes in cAMP metabolism. ANP had no effect on basal cAMP levels in GH₄C₁ cells or on adenylyl cyclase stimulation by vasoactive intestinal peptide (VIP) (Fig. 3a). Furthermore, 8-bromo-cGMP, which does not alter phosphodiesterase activity in other preparations^{4,5,16}, increased outward current just as effectively as ANP (Fig. 1c), even in the presence of 500 μM IBMX ($n=2$, not shown) or a hydrolysis-resistant analogue of cAMP (Fig. 3b). Finally, cGMP increased BK channel activity in cell-free patches where cAMP accumulation should be negligible (Fig. 3c). Direct effects of cGMP on BK channel activity¹⁸ were also excluded. When a pH-independent buffer (1 mM BAPTA, $p\text{Ca}=7$) was used to control the concentration of Ca^{2+} on the cytoplasmic side of the membrane, 100 μM cGMP had no effect on BK channel activity in the absence of ATP (Fig. 3d, $n=0/6$).

Two experiments showed that cGMP stimulated BK channel activity through activation of a cGMP-dependent protein kinase (PKG). First, application of the purified, cGMP-independent, catalytic fragment of the kinase to the cytoplasmic side of cell-free patches rapidly stimulated BK channel activity (Fig. 3e). On average, activity increased 350% within 10 minutes ($n=5/7$). Neither vehicle alone ($n=3$) nor the heat-inactivated enzyme ($n=3$) altered BK channel activity. Second, in cell-free patches BK channel stimulation by cGMP was blocked reversibly by 0.6 μM H8 (Fig. 3f, $n=3/4$) or 0.3 μM KT5823 ($N=2/2$, not shown), two PKG inhibitors¹⁹. Although these inhibitors also block the cAMP-dependent protein kinase (PKA) at higher concentrations¹⁹, cAMP-dependent phosphorylation inhibits BK channel activity in these cells (Fig. 4)^{11,20}. Therefore, inhibition of PKA by H8 or KT 5823 would stimulate BK channel activity, the opposite of what was observed.

The preceding results strongly support the conclusion that the effects of cGMP are mediated by an endogenous cGMP-dependent protein kinase. If the kinase had phosphorylated BK channels directly, one would have expected phosphatase inhibitors to potentiate cGMP action. But the effects of ANP and 8-bromo-cGMP on outward current in metabolically intact cells (Fig. 1b, c) and the effect of cGMP on cell-free patches (Fig. 4a, b) were blocked completely by 10 nM okadaic acid. At that concentration, okadaic acid is a selective inhibitor of protein phosphatase 2A (ref. 13); concentrations up to 1 μM had no effect on PKG activity (T.M.L., unpublished observa-

tions) or on BK channel activity in the absence of ATP¹¹. Furthermore, it is unlikely that okadaic acid blocked the effect of PKG by protecting a PKA site on the channel because cGMP was equally effective in the presence of 100 μM cAMP, which inhibited BK channel activity just as completely as okadaic acid (Fig. 4a, b, $n=3$). Finally, PKG did not stimulate BK channels by inhibiting PKA directly. As shown in Fig. 4c, addition of 300 nM wiptide, the polypeptide fragment of the endogenous skeletal muscle inhibitor protein for cAMP-dependent kinase²¹, to the cytoplasmic side of cell-free patches blocked cAMP action completely but did not mimic cGMP action ($n=4$). Furthermore, subsequent addition of cGMP stimulated activity with wiptide present ($n=3/4$). Therefore, we conclude that PKG stimulates BK channels indirectly through protein dephosphorylation.

To explain these results we suggest that cGMP-dependent protein kinase stimulates protein phosphatase 2A activity by phosphorylating the enzyme or one of its regulatory subunits.

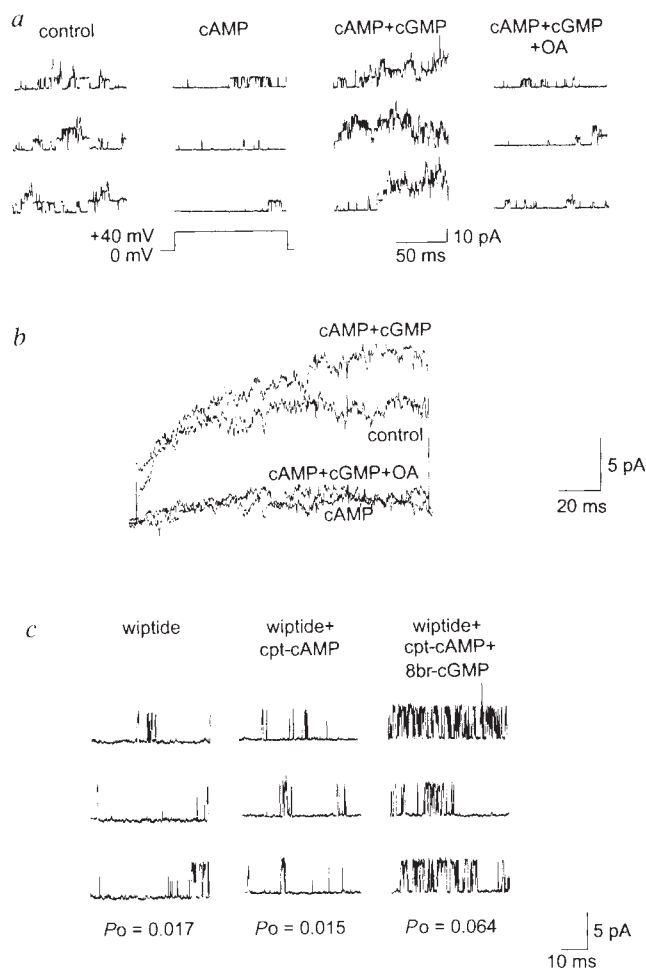


FIG. 4 Cyclic GMP stimulates BK channel activity through protein dephosphorylation. **a**, Representative records of unitary BK currents from an outside-out patch. Perfusion of the cytoplasmic surface of the patch with 100 μM cAMP inhibited channel activity, but addition of 100 μM cGMP to the perfusate reversed this effect. Okadaic acid (OA; 10 nM) completely blocked the stimulation of channel activity produced by cGMP. **b**, Ensemble averages of 100 consecutive single-channel records obtained under the experimental conditions illustrated in **a**. **c**, Representative records from another outside-out patch, to which 300 nM wiptide (Peninsula Labs) had been added to the pipette solution. This completely blocked the response to bath application of 0.1 mM 8-chlorophenylthio-cAMP (cpt-cAMP) but not the response to 1 mM 8-bromo-cGMP.

METHODS. Outside-out patches were formed and BK channel activity was elicited as described in Fig. 3. The averaged responses to 100 consecutive depolarizations are displayed in **b**.

Although biochemical studies on purified enzymes will be required to rule out more complicated alternative mechanisms, it is well established that protein phosphatase 2A modulates both BK channels and the dihydropyridine-sensitive Ca^{2+} channels^{22,23}. Thus phosphatase activation could also explain the inhibitory effect of ANP on dihydropyridine-sensitive Ca^{2+} channels (Fig. 1d-f)^{7,8}, which require cAMP-dependent phosphorylation to respond to membrane depolarization²⁴⁻²⁷. By simultaneously inhibiting Ca^{2+} channels and activating potassium channels, ANP could profoundly reduce electrical excitability. In fact, protein phosphatase activation by the cGMP-dependent protein kinase provides a new molecular mechanism for the antagonistic effects of cAMP and cGMP in many tissues, one that was anticipated almost 20 years ago in the yin-yang theory of Goldberg *et al.*¹². □

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Human TNF mutants with selective activity on the p55 receptor

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THE remarkable ability of tumour necrosis factor (TNF), especially in combination with interferon, selectively to kill or inhibit malignant cell lines is so far unmatched by any other combination of cytokines¹⁻⁴. But clinical trials in cancer patients have on the whole been disappointing⁵⁻⁷, and it has been estimated that a TNF dose would be effective only at 5-25 times the maximum tolerated dose⁴. High TNF concentrations give a much more pronounced antitumour activity in mice^{1,8-10}, in which murine TNF is about 50-fold more systemically toxic than human TNF^{11,12}. But there is little or no species specificity in cytotoxicity of murine TNF and human TNF on human as well as on murine cell lines^{13,14}. This dual action of TNF may be explained by the existence of two types of receptor for TNF^{15,16}: the smaller, TNF-R55, is present on most cells and particularly on those susceptible to the cytotoxic action of TNF¹⁷; the larger, TNF-R75, is also present on many cell types^{15,16}, especially those of myeloid origin, and is strongly expressed on stimulated T and B lymphocytes¹⁸. In mice, human TNF binds only to murine TNF-R55 (ref. 15), which can then mediate cytotoxic activity on malignant cells^{15-17,19}. As human TNF does not bind to murine TNF-R75, the latter must be responsible for the much enhanced systemic toxicity of murine TNF. Human TNF can, however, become toxic in mice when a second pathway is activated^{1,11,20}. There is no reciprocal situation in the human system: human and murine TNF bind almost equally well to the two human TNF receptors. Here we describe human TNF

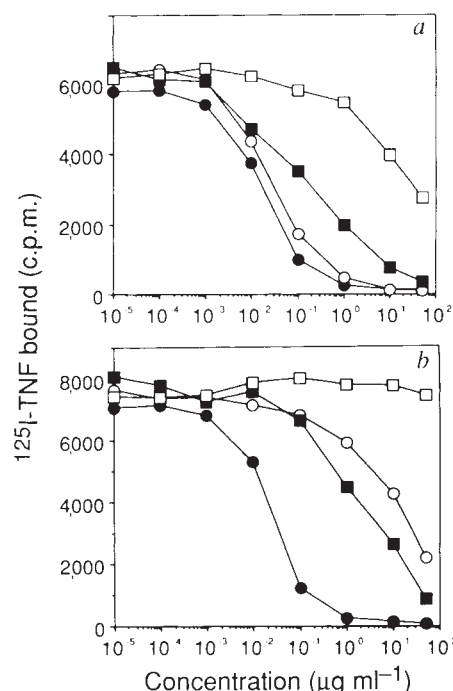


FIG. 1. Competitive binding of hTNF and mutants to recombinant hTNF-R55 and hTNF-R75. Microtitre plates coated with recombinant hTNF-R55-IgGγ3 fusion protein (a) and recombinant hTNF-R75-IgGγ3 fusion protein (b) were incubated with radiolabelled hTNF in the presence of different concentrations of competing wt hTNF (●), L29S (■), R32W (○) or A84V (□).

METHODS. hTNF-R55-IgGγ3 and TNF-R75-IgGγ3 fusion proteins²⁶ dissolved in phosphate-buffered saline (PBS) at 0.1 μg ml⁻¹ and 0.3 μg ml⁻¹, respectively, were added to microtitre plates (100 μl per well) and incubated overnight at 4 °C. Blocking was with 50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% NaN₃, and 1% defatted milk powder. The microtitre plates were then washed with PBS, and incubated with 10 ng ml⁻¹ 125I-labelled wt hTNF (specific radioactivity ~1.1 MBq μg⁻¹) in the presence of increasing concentrations of wt hTNF or mutant TNF. Total volume was 100 μl per well and each concentration was assayed in triplicate. After 3 h at room temperature, wells were washed with PBS and bound radioactivity was measured in a γ-counter.

mutants that still interact with the human TNF-R55 receptor but which have largely lost their ability to bind to human TNF-R75. Activation of TNF-R55 is sufficient to trigger cytotoxic activity towards transformed cells. One representative human TNF mutant retains its antitumour activity in nude mice carrying tumours derived from human cancers. Under the appropriate conditions, such human TNF mutants are expected to induce less systemic toxicity in man, while still exerting their direct antitumour effect.

We and others have previously characterized a number of human (h)TNF mutants which had lost almost all activity in the standard cytotoxicity assay with the L929 murine fibrosarcoma cell line^{21,22}. These point mutations, scattered throughout the amino-acid sequence, defined a restricted locus on the trimeric TNF molecule, corresponding to the region around the lower part of each cleft between two subunits. The threefold symmetry of the receptor-binding sites suggests that clustering is the basic mechanism of action of TNF. There is no *a priori* reason why an hTNF mutant, which has lost its binding capacity to mouse (m)TNF-R55 (that is, loss of bioactivity on L929 cells), should also have a modified binding affinity to one or the other of the two hTNF receptor types. Hence, we characterized a collection of hTNF mutants further by investigating their binding to and activity on both hTNF receptor types. Two of the mutants were found to have selectively lost most of their binding capacity to TNF-R75; these carried a Leu→Ser mutation at position 29, and an Arg→Trp mutation at position 32 (mutants L29S and R32W, respectively). For comparison, we use the inactive mutant Ala→Val at position 84 (A84V).

HEp-2, a human larynx carcinoma-derived cell line, only carries TNF-R55¹⁷ and is susceptible to the cytotoxic action of TNF in the presence of cycloheximide. Table 1 shows that L29S and R32W have largely retained the cytotoxicity of wild-type

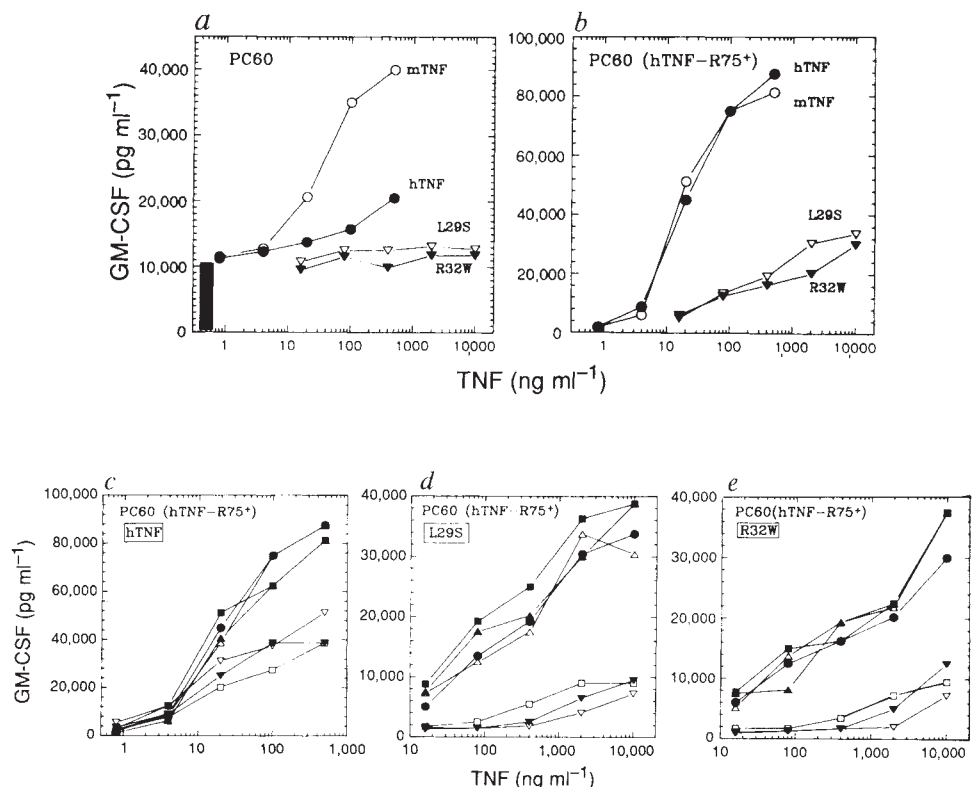
TABLE 1 Affinity and activity of hTNF mutants on HEp-2, U937 and L929 cells

TNF	Activity (U mg ⁻¹)	HEp-2 Binding K _D	U937 Binding K _D	L929 Activity (U mg ⁻¹)
wt	2.9(±0.2) × 10 ⁷ (100%)	9.2 × 10 ⁻¹⁰ (100%)	2.5 × 10 ⁻¹⁰ (100%)	2.6(±0.5) × 10 ⁷ (100%)
L29S	9.3(±7.4) × 10 ⁶ (32%)	1.1(±0.8) × 10 ⁻⁹ (86.5%)	5.1(±1.8) × 10 ⁻⁸ (0.5%)	2.2(±1.6) × 10 ⁵ (0.9%)
R32W	4.5(±3.5) × 10 ⁷ (155%)	1.1(±0.4) × 10 ⁻⁹ (86.5%)	3.5(±2.1) × 10 ⁻⁸ (0.7%)	6.6(±0.2) × 10 ⁴ (0.3%)
A84V	≤ 5 × 10 ³ (≈ 0.02%)	3.6(±2) × 10 ⁻⁸ (2.6%)	9.3(±1.1) × 10 ⁻⁹ (2.7%)	≤ 6 (< 0.01%)

Biological activity was assayed on human HEp-2 cells, which exclusively express TNF-R55, and on the standard murine cell line L929. Binding of wild-type hTNF on the two human cell lines was determined by Scatchard analysis. All other K_D values were found by competition analysis. Each value is the mean of at least two independent experiments. Relative values (taking wt TNF as 100%) are indicated in brackets. The standard procedure for TNF titration on L929 cells was followed³. The same procedure was used for HEp-2 cells, except that 50 µg ml⁻¹ cycloheximide was added instead of actinomycin D. The indicated values (with standard deviations) are the average of two independent experiments. For receptor binding experiments, hTNF was iodinated with Na¹²⁵I using lodogen (Pierce Chemicals) as oxidant. A specific radioactivity of 2.8 MBq µg⁻¹ was obtained. There was no significant reduction in bioactivity of iodinated TNF. For Scatchard analysis, a dilution series (12.8 nM to 0.006 nM) of the labelled TNF was prepared in triplicate in a microtitre plate (based on an M_r of the subunit of 17,350). Nonspecific binding was measured by addition of a 100-fold excess of unlabelled TNF to each well; about 2 × 10⁶ cells were added. The reaction was done in 0.2 ml cell culture medium supplemented with 0.1% NaN₃, and kept for 2–3 h at 4 °C. Cells were then separated from the medium by centrifugation through 300 µl phthalate oil (33% dinonylphthalate and 66% dibutylphthalate (v/v)) and the cell-associated radioactivity measured. A Scatchard plot and K_D values were determined using an EBDA/LIGAND programme (Elsevier Biosoft, Cambridge, UK). For competition experiments, a 1:2 dilution series of unlabelled mutant TNF (2,000 nM to 4 nM) was set up over 10 wells in a microtitre plate. The two remaining wells contained only labelled hTNF (total binding) and a 5,000-fold excess of unlabelled wild-type hTNF (background), respectively. To all wells, 0.4 nM of ¹²⁵I-labelled wtTNF was added. After addition of 2 × 10⁶ cells, the total volume was 0.2 ml per well.

FIG. 2 Induction of granulocyte macrophage colony-stimulating factor (GM-CSF) by mTNF, hTNF or hTNF mutants L29S and R32W. A dose-response curve for mTNF, hTNF, L29S and R32W for their capacity to induce GM-CSF secretion in parental and in PC60 hTNF-R75⁺ cells (clone 26) is shown in *a* and *b*, respectively. The effect of neutralizing anti-hTNF-R75 (Utr-1, ▽; Utr-2, ▼; Utr-3, □), non-neutralizing anti-hTNF-R75 (Utr-4, ■; Utr-10, △) and an irrelevant monoclonal antibody (Htr-9, ▲; anti-hTNF-R55), as compared to control (●), on wt hTNF-, L29S- and R32W-dependent induction of GM-CSF in PC60 hTNF-R75⁺ cells (clone 26), is shown in *c*, *d* and *e*, respectively. Note that the co-ordinate scales are not identical in *a*–*e*.

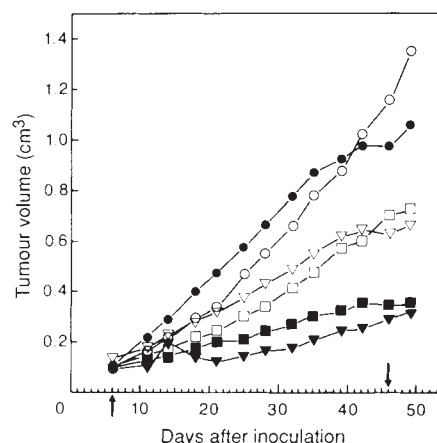
METHODS. Induction experiments were done in 96-well microtitre plates using 3 × 10⁴ PC60 or PC60 hTNF-R75⁺ cells per well. As a 50-fold synergism was observed between TNF and the combination of IL-1/IL-2 both in parental and in transfected PC60 cells, all induction experiments were done in the presence of IL-1 (1 ng ml⁻¹) and IL-2 (100 IU ml⁻¹). For the experiments in *c*, *d* and *e*, the PC60 hTNF-R75⁺ cells were pretreated for 1 h at 37 °C with the indicated monoclonal antibodies at a concentration of 2 µg ml⁻¹, after which dilutions of hTNF, L29S or R32W, respectively, were added. Following 36 h incubation, the supernatant was assayed for GM-CSF activity by its capacity to induce proliferation of FDCpl cells²⁴. Isolation of PC60 hTNF-R75⁺ cell clones: PC60 cells²⁷ were electroporated with pSV25hTNF-R75, a eukaryotic expression vector containing the coding region of the hTNF-R75 gene¹⁶ under control of the SV40 early promoter, together with pBSΔppac, a selection plasmid



containing the gene for *N*-acetylpuromycin transferase²⁸. Transfected cells were continuously selected in 3 µg ml⁻¹ puromycin, and subcloned by limiting dilution. Cloned cells were stained with Utr-1 and fluoresceinated goat anti-mouse immunoglobulin (Sera-Lab, Crawley Down, UK), and analysed by flow fluorocytometry (Epics 753; Coulter). A typical transfectant (clone 26) was used for all further experiments.

FIG. 3 Comparison of the antitumour activity of wt hTNF and R32W, without or with added hIFN- γ , in nude mice carrying a human HT-29 tumour.

METHODS. HT-29, a human colon adenocarcinoma cell line, was obtained from the ATCC (Rockville) and cultured in DMEM, supplemented with 10% FCS, 2 mM L-glutamine, 50 IU ml⁻¹ penicillin G and 50 μ g ml⁻¹ streptomycin sulphate. After washing with PBS, 5×10^6 cells were injected s.c. in the backs of 10-week-old female nu/nu (nude) mice (Iffa-Credo, Saint Germain-sur-l'Arbresle, France). Treatment started on day 6 after inoculation (upward-pointing arrow) and comprised 6 daily perilesional injections, followed by 1 day of rest, for 6 weeks (last injection indicated by downward-pointing arrow). The tumour volume was estimated every 3 or 4 days by measuring the larger (x) and the smaller (y) perpendicular diameters, and was calculated according to the formula $x \times y^2 \times 0.4$. Means of 5 mice per group are plotted. Mice received either recipient buffer (PBS) alone ($\circ$), 10 μ g wt hTNF (∇), 10 μ g R32W ($\square$), 6.7×10^3 IU hIFN- γ ($\bullet$), 10 μ g wt hTNF and 6.7×10^3 IU hIFN- γ ($\blacktriangledown$), or 10 μ g R32W and 6.7×10^3 IU hIFN- γ ($\blacksquare$), in 0.1 ml volume. Statistical analysis was done using the Mann-Whitney U-test; there was no significant difference between the wt hTNF and R32W treatments, either alone or in the presence of IFN- γ . The wt hTNF and R32W preparations were >99% pure and contained <2.5 ng per mg endotoxin. The hIFN- γ preparation had a specific activity of 2.5×10^7 IU mg⁻¹.



(wt) TNF. Furthermore, binding measured by displacement of ¹²⁵I-labelled wtTNF indicates that the two mutants have almost unaltered receptor affinity. On the other hand, U937, a human promyelocytic cell line, expresses mainly hTNF-R75 and also some hTNF-R55 (ref. 17). As hTNF binds with about 5-fold higher affinity to the larger receptor^{15,16}, suboptimal concentrations of ¹²⁵I-wtTNF are predominantly bound to hTNF-R75. Under these conditions, L29S and R32W have virtually lost the capacity to compete with this binding (Table 1).

We repeated the binding studies using purified, recombinant hTNF receptors. The results (Fig. 1) independently confirm those shown in Table 1. From the values at 50% binding, one can deduce that the affinity of L29S relative to wtTNF is lower by factors of 8.5 and 115 for hTNF-R55 and hTNF-R75, respectively. The decrease in affinity of the R32W mutant amounts to factors of 1.4 and 615, respectively. The latter mutant thus has practically retained its binding properties to hTNF-R55, but largely lost its binding to hTNF-R75. The corresponding values for the reference mutant A84V are a decrease in affinity by a factor of 1,500 for hTNF-R55, and no detectable binding to hTNF-R75.

To assess hTNF-R75-mediated activity, we made use of the fact that murine T cells and lines carry only or predominantly mTNF-R75, and therefore respond to mTNF, but only weakly or not at all to hTNF. For example, mTNF, but not hTNF, induces in the rat/mouse thymoma line PC60 a set of proteins, such as the interleukin-2 (IL-2) receptor- α and granulocyte macrophage colony-stimulating factor (GM-CSF)^{23,24}. The results (Fig. 2a–b) show that PC60 cells transfected with the hTNF-R75 gene (PC60 hTNF-R75⁺) now responded to hTNF to the same extent as to mTNF. In contrast, the hTNF mutants L29S and R32W only gave a very weak response. To confirm that the observed effect was mediated by hTNF-R75, we did control experiments using Utr monoclonal antibodies¹⁷ directed against hTNF-R75. Utr-1, -2 and -3 compete with hTNF for receptor binding, whereas Utr-4 and -10 react with the same antigen but do not compete, and therefore do not inhibit in the assay (Fig. 2c). The residual activity in the assay with the mutants (Fig. 2b) was due to weak interaction with hTNF-R75 and could be suppressed by the neutralizing antibodies Utr-1, -2 and -3 (Fig. 2d–e, respectively). These results indicate that the two hTNF mutants have some residual biological activity on hTNF-R75, but it is about 100-fold less than that of wild-type hTNF.

As the mutants L29S and R32W retained cytotoxic activity in human cell culture, we tested whether the latter mutant had antitumour activity *in vivo*. HT-29 is a cell line derived from human colon adenocarcinoma, which *in vitro* is sensitive to TNF in the presence of interferon (IFN)³. HT-29 tumours were

grown in nude mice and when the nodules became palpable, daily treatment was started (Fig. 3). Administration of either wtTNF or mutant R32W resulted in a clear retardation of tumour growth. Furthermore, a combination of hTNF with hIFN- γ almost completely inhibited growth of the tumour and some animals were apparently cured, although tumour growth started again after treatment was stopped (this was not surprising in view of the fact that nude mice lack cellular immunity). Also there was no significant difference in antitumour activity between wtTNF and R32W in combination with IFN- γ . As expected, there were no treatment-related deaths in any of the groups. Similar results were obtained with another human tumour-derived cell line, ME-180.

The results obtained with hTNF as opposed to mTNF in experimental tumour systems in mice indicate that antitumour activity and systemic toxicity of TNF are at least partially based on different mechanisms. Similarly, the finding that experimental animals can be tolerized against systemic TNF toxicity without compromising antitumour efficacy of TNF combined with IFN¹⁰, indicates that the two activities may be dissociable. In some cells, however, triggering TNF-R75 may also contribute to the direct cytotoxic pathway²⁵. Furthermore, hTNF can be toxic and even lethal to mice under certain conditions, for example when a sensitizing agent, such as IL-1, lipopolysaccharide or a glucocorticoid antagonist, is present^{1,11,20}. These observations suggest that the mechanism(s) of systemic toxicity is (are) distinct and therefore potentially amenable to specific inhibition.

In conclusion, it remains that (1) hTNF, which only binds to mTNF-R55, is almost non-toxic for normal mice; (2) in the human system, the mutants R32W, and to a lesser extent L29S, practically do not bind to hTNF-R75, but retain their activity on hTNF-R55; (3) the R32W mutant still has a direct antitumour activity *in vivo*. Further experiments will verify whether hTNF-R55-specific TNF mutants reduce systemic toxicity in primates as they do in murine systems. If so, they may offer new opportunities for cancer treatment, based on the direct antitumour activity of the TNF mutants, preferably in combination with interferons or other synergistic chemotherapeutic drugs. □

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Right-handed rotation of an actin filament in an *in vitro* motile system

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MUSCLE contraction occurs by mutual sliding between thick (myosin) and thin (actin) filaments^{1,2}. But the physical and chemical properties of the sliding force are not clear; even the precise direction of sliding force generated at each cross-bridge is not known. We report here the use of a recently developed *in vitro* motile assay system³⁻⁵ to show supercoiling of an actin filament in which the front part of the filament was fixed to a glass surface through cross-linked heavy-meromyosin and the rear part was able to slide on a track of heavy-meromyosin. A left-handed single turn of superhelix formed just before supercoiling, suggesting that the sliding force has a right-handed torque component that induces the right-handed rotation of an actin filament around its long axis. The presence of the torque component in the sliding force will explain several properties of the contractile system of muscle.

When the sliding velocity of the front part of an actin filament is lower than that of the rear part, the central part can become buckled^{6,7}. The buckled part is then transformed into a single turn of superhelix and then into a loop⁷ (we call this a superhelix, because the actin filament itself has a helical symmetry). When the front part stops sliding, the rear part wiggles like a fishtail, as previously predicted⁸. Such phenomena were interpreted as being due to the presence of a torque component in the sliding force which rotates the filament around its long axis. But the handedness of the torque could not be determined, mainly because both right- and left-handed superhelices appeared⁷.

To determine the handedness of the torque component, we designed a new artificial *in vitro* motile system (Fig. 1), in which the front part of the actin filament was attached to a glass surface through undissociable heavy meromyosin (HMM) track⁹. We

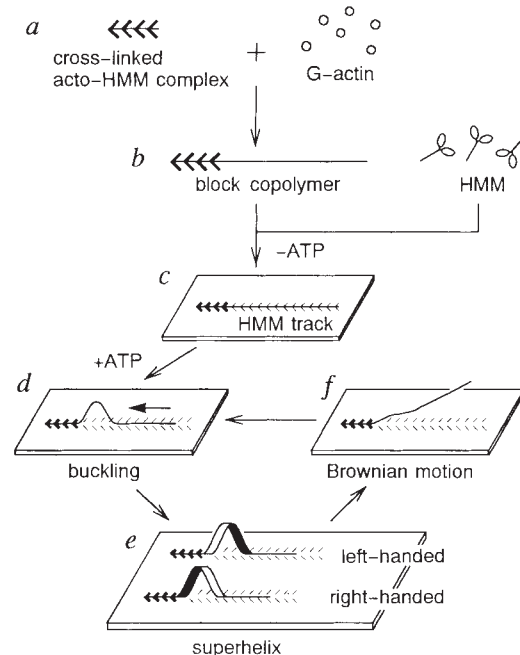
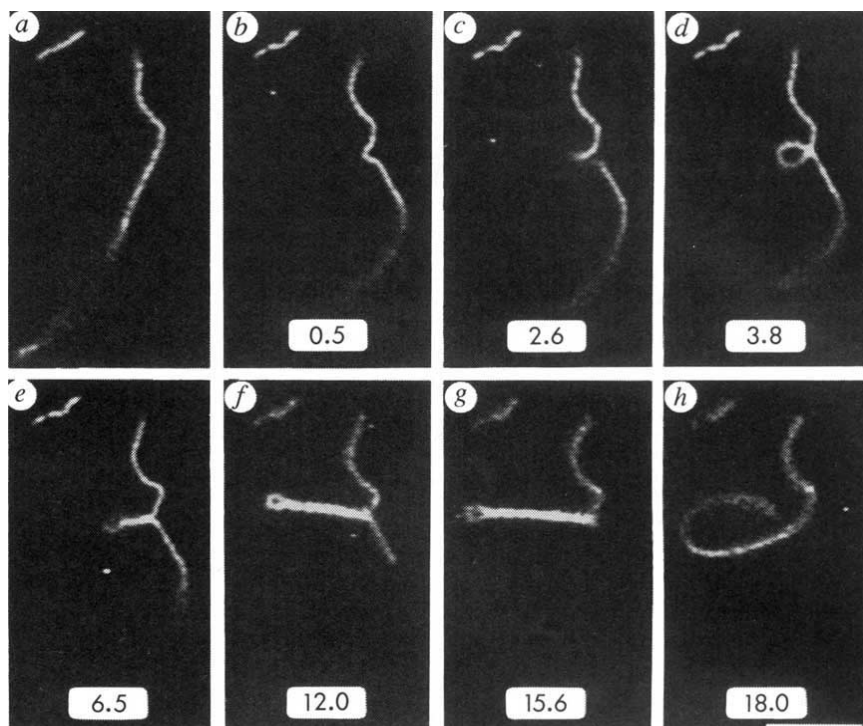


FIG. 1 A flow chart illustrating the arrangements of the artificial *in vitro* motile system which was designed to determine the handedness of torque component of the sliding force (for details on a-f, see below and text). Actin and myosin were prepared from the back and leg white skeletal muscle of a rabbit according to a standard procedure¹⁰. HMM was prepared by chymotryptic digestion of myosin¹⁵ without ammonium sulphate fractionation, stored in liquid N₂ and used within a few days after quick thawing. Acto-HMM complexes covalently cross-linked were prepared by treating the F-actin-HMM (1:4 weight ratio) complexes with 3 mM 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC; Nakarai Tesque Co. Ltd., Kyoto) under a rigor condition following the techniques of Ando¹⁶ with slight modifications. Acto-HMM complexes chemically modified were prepared by mixing F-actin and *N*-ethylmaleimide (NEM)-treated HMM in a 1:4 weight ratio. The results were indistinguishable between the two types of acto-HMM complexes. The growing type of block copolymers were prepared as follows (b): either one of the acto-HMM complexes prepared as described above was first diluted to 0.6 mg ml⁻¹ in solution A composed of 25 mM KCl, 2 mM MOPS (pH 7.0), 0.5 mM MgCl₂ and 1.5 mM NaH₂PO₄; the complexes were fragmented by sonication for 30 s at 0 °C in an insonator at 9 kHz and 100 W (Model 200M, Kubota Co., Tokyo). G-actin diluted in solution A to a final concentration of 0.12 mg ml⁻¹ was added to the solution of acto-HMM fragments immediately after the dilution and preferentially polymerized onto the barbed end of the fragments at room temperature¹⁰ (a, b). Fifteen minutes later, the solution was diluted to 0.1 M KCl, 2 mM MgCl₂ (finally, 0.1 mg ml⁻¹ actin) and 45 min later, 3.6 μM rhodamine phalloidin (Rh-Ph, Molecular Probes, Eugene, OR) was added and stored on ice. An *in vitro* motility assay was done according to Toyoshima *et al.*⁵ with slight modifications as follows: the nitrocellulose-coated glass cover was prepared by drying it after dipping it in 0.1% collodion. The Rh-Ph labelled block copolymers (0.05 mg ml⁻¹ actin; b) were mixed with HMM (0.38 mg ml⁻¹) in 0.1 M KCl and 2 mM MgCl₂ and stored for 20 min at room temperature; this solution was then diluted 100 times with solution B composed of 25 mM KCl, 25 mM imidazole-HCl (pH 7.4), 4 mM MgCl₂ and 1 mM EGTA and put in a flow cell made of a pair of cover slips with a spacer 75 μm thick and sealed with white petrolatum (c). After 30-60 s, the flow cell was rinsed with solution B with the addition of 0.5 mg ml⁻¹ BSA (Sigma) and free proteins were washed out with solution B with the addition of 10 mM DTT and the oxygen-depleting enzyme system (0.22 mg ml⁻¹ glucose oxidase (Sigma), 0.036 mg ml⁻¹ catalase (Sigma) and 4.5 mg ml⁻¹ glucose)¹⁷. The sliding was initiated by flowing solution B with the addition of 4 mM ATP, 10 mM DTT and the oxygen-depleting enzyme system (d-f; the sliding direction is shown by an arrow) and observed under an inverted fluorescence microscope (Diaphoto-TMD; NCF Fluor ×100 objective lens (numerical aperture (NA)=1.3); Nikon, Tokyo) equipped with a highly sensitive TV camera (SIT camera (C1000-12) with a contrast enhancer (M1438), Hamamatsu Photonics, Hamamatsu, Japan). The fluorescent images were recorded on a video-tape recorder (SR5050, Victor, Tokyo). The sense of the superhelix was determined following the methods of Shimada *et al.*¹⁸, remembering that the image obtained under the inverted microscope we used was the mirror image of the real one. That is, in the superhelical part of e, each white part on this side is out-of-focus, while each black part on the glass surface is focused.

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FIG. 2 An example showing supercoiling of an actin filament. A sequence (a–h) of fluorescent images of an actin filament, of which the front part, corresponding to the pointed end, is attached to a glass surface through cross-linked HMM molecules and the rear part slides on an HMM track. The figures on each panel indicate time (s) after the rear part attached itself to the HMM track. The small circle of supercoil rotated at least four times during the sliding of the rear part by about $5\text{ }\mu\text{m}$, but it was difficult to determine the number of rotations precisely. Throughout the above process, the sliding velocity of the rear part was $1\text{ }\mu\text{m s}^{-1}$ on the average and changed within a factor of 2, irrespective of whether supercoiling occurred or not. Such a series of supercoiling repeated up to 7 times on the same HMM track and stopped for one of the following reasons: (1) the junction between the front part, covered with undissociable HMM, and the buckled part was broken; (2) the small circle of supercoil was broken, probably because of the accumulation of strain for both cases (1) and (2); or (3) the rear part stopped sliding on the HMM track. The buckled part was always transformed into a loop through a single turn of superhelix, but the supercoiling did not always occur. The occurrence frequency of supercoiling was about 20%, and in the remaining 80% only the radius of the loop increased, without supercoiling. We have 94 supercoils so far, among which 71 cases showed a left-handed superhelix, 2 cases a right-handed one and the remaining 21 cases were undetermined. In the superhelices which were not transformed into a supercoil (about 350 cases total), the number of superhelices for which handedness could be determined was 277, of which 251 cases (91%) were



left-handed and 26 cases were right-handed (9%). About 80% of these data were obtained from cross-linked acto-HMM block copolymers. For more details, see text. Scale bar, $5\text{ }\mu\text{m}$.

first prepared the acto-HMM complexes either covalently cross-linked or chemically modified to make the HMM undissociable in the presence of ATP. After fragmentation of these complexes, actin was polymerized preferentially onto the barbed end of the fragments. Then this growing type of block copolymers¹⁰ was covered with HMM so they could be used as the HMM track⁹. We expected that the handedness of torque component, which

induces the rotation of the filament, must be determined by the helical sense of the superhelix to be formed at the central part of the filament (Fig. 1e).

A typical result is shown in Fig. 2. Owing to the continued sliding of the rear part of the filament, the central part was buckled (Fig. 2b) and then transformed into a left-handed superhelix (Fig. 2c), as schematically illustrated in Fig. 3a. After the superhelix was transformed into a loop (Fig. 2d), the loop was unexpectedly transformed further into a multifold superhelix (Fig. 2e; we call this a supercoil). The supercoiled part sticking out sideways was composed of a small circle at the tip and a rod part. The length of the rod part increased with time (Fig. 2e–g) and the increase in length was nearly half the distance that the rear part slid. In addition, the fluorescence intensity of the rod part was much higher than that of the other parts, consistent with the rod part being composed of doubled filaments, as schematically illustrated in Fig. 3b. Finally, the rear part of the filament was detached from the HMM track,

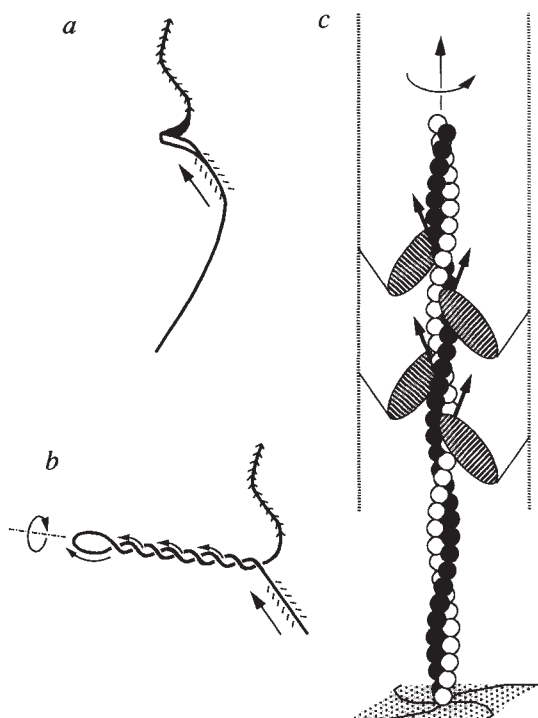


FIG. 3 Schematic illustration of a left-handed superhelix (a) and a supercoil (b) of actin filaments corresponding to Fig. 2c and f, respectively, and the situation expected in a sarcomere (c). Thin arrows indicate the direction of sliding and rotation. c. It is assumed that one end of the thin filament is fixed at the Z-line, whose lattice structure is schematically illustrated at the bottom, whereas the rest, including the end shown at the top, is free to rotate. For simplicity only a single head of myosin (the ellipsoids with hatching) is drawn. The direction of sliding force imposed on a thin filament through each cross-bridge is shown by a thick arrow. The reaction force having a direction opposite to the thick arrow will be imposed on each myosin head, which may induce the compression of the filament lattice. Although the direction of the sliding force may be nearly parallel to the long helical strand of the actin filament (the open or closed circles), as shown here, the direction of the resultant force will change depending on the external force (load) and its moment.

and this was followed by an unwinding of the supercoil and then a brownian motion (Fig. 2a, h). When the rear part reattached to the HMM track, the same process started again (compare with Fig. 1d-f).

Judging from the fact that the superhelices formed just before supercoiling were left-handed in almost all cases (71 out of 73 cases), we conclude that the sliding force contains a right-handed torque component, which induces rotational sliding of an actin filament in the manner of a right-handed screw. (A microtubule also shows a right-handed screw movement on dynein attached to the glass surface¹¹.) In the exceptional right-handed superhelices (2 out of 73 cases), the rapid transformation from right- to left-handed may have occurred just before supercoiling; the reason we infer this is that, in the superhelices which were not transformed into a supercoil (about 350 cases), there were 4 cases in which a loop once formed through a right-handed superhelix was transformed into a left-handed one, but no reverse cases. In addition, there is a possibility that a certain proportion of superhelices are simply formed by the compressive force produced by the sliding of the rear part of the filament (compare with ref. 7), but the supercoil will not be.

Whether the supercoiling occurs or not is probably determined by the balance between the magnitude of the net torque and the rigidity of the actin filament. It seems that an essential factor for supercoiling is to fix physically the front part while keeping the rear part sliding and to make the buckled part free to move without attaching extra HMM molecules. A particular arrangement of HMM molecules that may exist in the HMM track will not be indispensable for supercoiling. In fact, a 2-fold supercoil (the superhelix described above is a 1-fold supercoil) was also observed in the usual *in vitro* motile system, although only once. Thus, we conclude that the motive force for supercoiling is intrinsic to the sliding force generated at each cross-bridge.

In a muscle fibre, where an actin filament cannot rotate freely, a twist of thin filaments will accumulate, so that the elastic

energy of the twist will be stored in the filament lattice, including the Z-line under an isometric condition (compare with Fig. 3c). Under an isotonic condition, both twisting and untwisting of a thin filament may occur along the filament here and there owing to its flexibility. In any case, the right-handed torque component tends to make the pitch of a right-handed long helix of thin filaments smaller. Such a strain may be strong enough to transform the lattice structure of the Z-line under certain conditions^{8,12,13}. Also, the radial compression of a fibre observed with accompanying tension development¹⁴ may be partly attributable to the radial component of the sliding force (compare with Fig. 3c). Thus, several properties of the contractile system of muscle (and also non-muscle cells) will be explained by the presence of a right-handed torque component in the sliding force (and the resultant supercoil). □

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DNA polymerase- α is essential for mating-type switching in fission yeast

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IN the fission yeast *Schizosaccharomyces pombe*, the double-stranded chromosomal break (DSB) at the mating-type locus (*mat1*) initiates recombination during mating-type switching¹⁻³. A constant DSB level is maintained throughout the cell-cycle¹. In the strand-segregation model for mating-type switching, it was postulated that if the DSB is generated during or soon after *mat1* replication⁴, one of the chromatids could be repaired and switched during replication in the next cell cycle, while the other chromatid inherits the break³⁻⁶. Here we report a molecular characterization of *swi7*, one of the genes required for DSB formation. Surprisingly, a gene complementing the *swi7* mutation maps to chromosome I and encodes *S. pombe* DNA polymerase- α . Disruption of this gene is lethal in both switching and non-switching strains, as expected. *S. pombe* DNA polymerase- α must therefore play a role in generating the DSB at *mat1*, suggesting that DSB formation is coupled with DNA replication.

Mutations in the switch (*swi*) genes of *S. pombe*, *swi1*, *swi3*

and *swi7*, reduce the level of the double-stranded chromosomal break and, consequently, such mutants switch inefficiently³. The *swi7* gene was isolated by complementation of *swi7-1*, the only known mutation of *swi7*. The plasmid containing the 6.5-kilobase (kb) clone (Fig. 1) was integrated into the genome by homologous recombination in the *swi7-1* strain, and the site of integration was confirmed by Southern blot analysis (not shown). The transformants showed a normal level of the DSB, demonstrating complementation of *swi7-1* mutation (Fig. 2). When the resulting *Leu⁺*, *Spo⁺* transformant cells (*Spo⁺* implies efficiently switching and sporulating) were crossed with cells of another *Swi⁺*, *leu1⁻* strain, all of the 21 tetrads analysed generated a 2 *Leu⁺*, *Spo⁺*: 2 *Leu⁻*, *Spo⁺* segregation pattern, demonstrating that the cloned sequence was linked to the *swi7* locus.

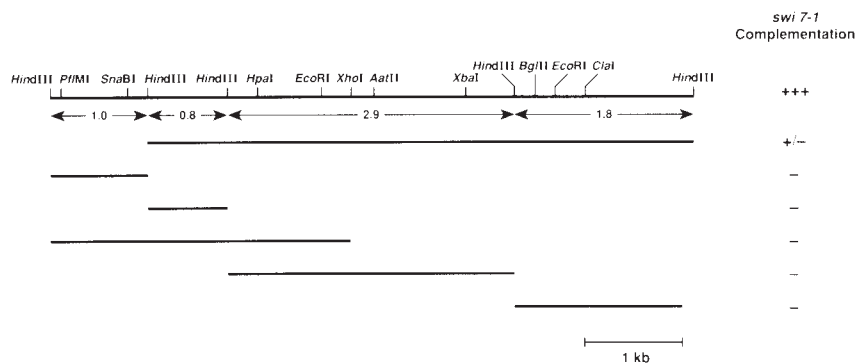
To identify the complementing activity, we determined the nucleotide sequence of the entire 6.5-kb clone (Fig. 1). An open reading frame encoding 1,405 amino acids spanning the 2.9 kb and 1.8-kb *HindIII* fragments was found. A database⁷ search revealed that the *swi7* gene is identical to the gene encoding DNA polymerase- α of *S. pombe*⁸ (data not shown).

To investigate how DNA polymerase- α might be involved in generating the DSB, we sequenced the *swi7-1* mutant allele. Transformation experiments first localized the mutation in the region between the *BglII* and the distal *HindIII* site (Fig. 1), and sequencing identified a mutation in codon 1,116, converting it from GGA (Gly)⁸ to GAA (Glu) (not shown). The *BglII/HindIII* fragment contains only part of the *pol* α ORF and corrects the *swi7-1* defect by homologous integration; this confirms that *pol* α is *swi7*. This mutation is not located in any of the regions known to be conserved in the DNA polymerases of different species⁹. Its location fails to suggest how DNA polymerase- α is involved in generating the DSB. Furthermore, a disruption

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FIG. 1 Restriction map of the *swi7* genomic clone and complementation results.

METHODS. We transformed SP509, a switching-defective strain (genotype: *h⁹⁰, leu1⁻, his2⁻, ade6⁻, swi5⁻, swi7⁻*; see below) with the *S. pombe* partial *HindIII* genomic library (kindly provided by P. Young and D. Beach) cloned in the vector pWH5 (ref. 23). The vector contains the *Saccharomyces cerevisiae* *LEU2* gene, which upon transformation complements the *leu1⁻* defect of *S. pombe*²⁴. Efficiently switching colonies contain cells of both mating types, which mate and sporulate. The spores contain a starch-like compound which stains black with iodine. Thus, the iodine staining phenotype of colonies is an easy assay for the efficiency of switching²⁴. The double mutant *swi5⁻, 7⁻* was used as it has a lower background level of staining with iodine than the single *swi7⁻* mutant and thus facilitated screening for better-staining transformants. The *swi5⁻* mutation affects the efficiency of switching but not the level of the DSB³. Of about 200,000 transformants screened, 47 stained darker with iodine vapours as compared to the parental strain. Of the 12 transformants tested, 10 complemented the DSB defect of the recipient strain. Plasmids recovered from the transformants restored the staining phenotype and the DSB level when transformed into another *swi7-1* mutant strain, SP112 (genotype: *h⁹⁰,*



swi7-1, leu1, ade6). The indicated *HindIII* fragments of the 6.5 kb clone were subcloned into the *HindIII* site of the vector pWH5, and the *XhoI-HindIII* fragment was cloned into the vector pIRT2 (ref. 25). DNA-mediated transformation was done according to ref. 24. Complete complementation is indicated by (+++), partial by (+/-) and (-) signifies lack of complementation. The sequence determined for *swi7* ORF was compared with the GCG Sequence Analysis Software Package, version 6 (ref. 7).

mutation caused cell inviability in both switching and non-switching strains (Fig. 3).

The *polα* gene had been mapped to chromosome II by pulse-field gel analysis⁸, but *swi7* had been genetically mapped about 9 centimorgans (cM) away from the *aro5* locus on chromosome I^{10,11}. We used genetic and physical studies to resolve this discrepancy. The genetic distance between *aro5* and the homologously integrated *polα* gene was calculated to be 8.0 cM¹² (42 parental ditype, 8 tetratype and no nonparental ditype tetrads), and a Southern blot of intact *S. pombe* chromosomes showed hybridization of the *polα* probe to chromosome I (Fig. 4a, lane 2) and to the *SfiI* fragment F (Fig. 4b), which was previously assigned to the region containing *swi7* and *aro5* on

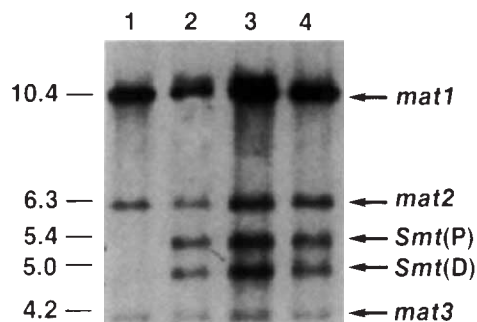


FIG. 2 The *polα* gene restores the DSB level in the *swi7-1* mutant. The autoradiograph shows the *HindIII*-digested DNA from the *swi7-1* strain SP112 (lane 1), two different SP112 transformants carrying the homologously integrated copy of the *polα* gene (lanes 2 and 3), and the *Swi⁺* wild type strain SP837 (lane 4; genotype: *h⁹⁰, leu1, ura4, ade6*) as a control. The DNA fragment sizes in kb are shown on the left and the *mat* loci are identified on the right. The 5.4- and 5.0-kb fragments labelled as *Smt(P)* and *Smt(D)* are generated by the DSB in the 10.4-kb *mat1*-containing fragment.

METHODS. The *swi7-1* mutant strain SP112 was transformed with a plasmid containing the whole genomic clone of *polα* that had been linearized at the unique internal *XhoI* site. Several stable *Leu⁺* transformants were obtained. DNA was prepared according to ref. 24 and digested with *HindIII*. Agarose gel electrophoresis, Southern blotting, probe labelling, hybridization and washing were done by standard methods²⁶. The blot was probed with the 10.4-kb *HindIII* fragment of *mat1* containing the *P* allele which was labelled with [α -³²P]dCTP by the random-primer labelling method.

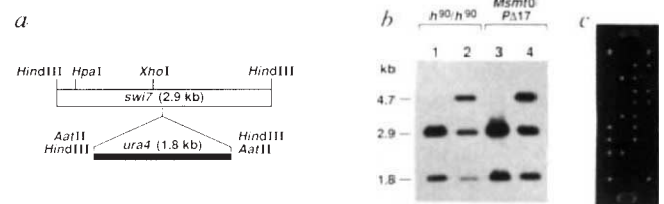


FIG. 3 Disruption of *swi7/pola* is lethal in both switching and non-switching haploid strains. **a**, The construct used for disruption of the *swi7/pola* gene. **b**, Southern blot analysis of diploids containing the heterozygous genomic disruption of *swi7/pola*. *HindIII* digests of DNA isolated from the parental *h⁹⁰/h⁹⁰, ura4⁻/ura4⁻* diploid (lane 1), with its heterozygous disruption (lane 2), and the non-switchable parental *mat1-Msmto/mat1-PΔ17::LEU2, ura4⁻/ura4⁻* diploid (lane 3), with its heterozygous disruption (lane 4) are displayed. The *mat1-Msmto²⁷/mat1-PΔ17::LEU2²⁸* diploid strain is a non-switching strain as it lacks the DSB due to deletion of essential *mat1*-cis-acting sequences. The parental diploids show the presence of the 2.9- and 1.8-kb *HindIII* fragments of the *swi7* gene (lanes 1 and 3), whereas the diploids carrying the heterozygous disruption show the additional predicted fragment of 2.9+1.8=4.7 kb (lanes 2 and 4). The ratio of intensity of the 2.9-kb to 1.8-kb band is reduced by half in the diploid containing the heterozygous disruption as compared to the parental diploid; this confirms the disruption within the 2.9-kb *HindIII* fragments of one of the chromosomes. **c**, The disruption of *swi7* is recessive lethal. Dissection of both heterozygous diploids presented in **b** gave a 2 live: 2 dead segregation pattern in all the tetrads analysed. All viable segregants were *Ura⁻*, indicating that the *Ura⁺* segregants containing the *polα* disruption die.

METHODS. The construction shown in **a** was made as follows. The 2.9-kb *HindIII* fragment was cloned into the *HindIII* site of Bluescript KS(+). The resulting DNA was cut with *AatII*, a unique site located in the *swi7* coding region and blunt-ended with T4 DNA polymerase. The DNA was then ligated to the 1.8 kb *HindIII* fragment of the *S. pombe ura4* DNA which had been blunt-ended with the Klenow fragment of DNA polymerase I. For the *in vivo* disruption shown in **b**, the plasmid DNA was cut with *HindIII*. The *ura4⁻/ura4⁻* diploid strains mentioned above were transformed with the cut DNA, selecting for *Ura⁺* prototrophs²⁴. DNA prepared from *Ura⁺* transformants was digested with *HindIII*. Agarose gel electrophoresis, Southern blotting, probe labelling, hybridization and washing were as described in the Fig. 2 legend. The 2.9- and 1.8-kb *HindIII* fragments were used together, in equimolar amounts, as the probe. **c**, The stable *Ura⁺* diploids were sporulated and dissected on plates containing rich medium. Four spores of each ascus were placed in a horizontal row. The photograph was taken after 3 days of growth. The uracil requirement and sporulation phenotypes of the segregants were tested by replica plating on to plates containing the appropriate medium²⁴.

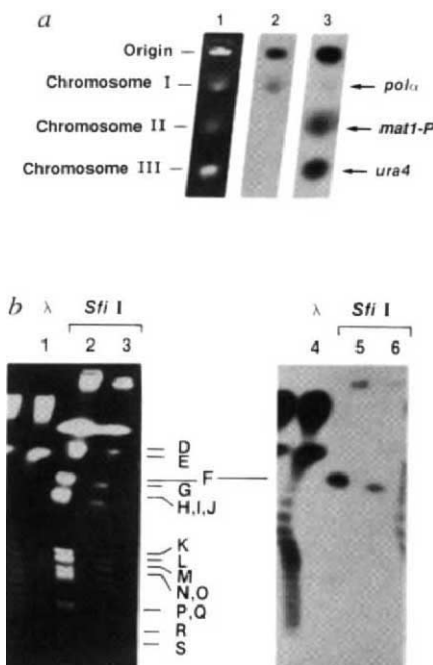


FIG. 4 *swi7/pola* is located on chromosome I within the *Sfi*I fragment F. METHODS. Both the blots and the photograph of the stained gels were kindly provided by C. Smith and J. B. Fan. The conditions for pulse-field electrophoresis and blotting have been described¹³. a, A blot of the intact *S. pombe* chromosomes was probed first with the *swi7* 1.8 kb *Hind*III fragment and autoradiographed showing hybridization to chromosome I (lane 2). For reference, the same filter was hybridized with a mixture of probes made from *mat1-P* (chromosome II) and *ura4* (chromosome III; lane 3) and autoradiographed again. Lane 1 shows the ethidium bromide-stained gel containing intact chromosomes. The top band ('origin') represents total DNA which is usually retained in the well in this gel system. b, The blot of the *Sfi*I digests of the chromosomes was probed with the 1.8-kb *Hind*III fragment of *swi7*. Lanes 1–3 show the ethidium bromide-stained gel containing λ -multimer ladder markers (lane 1) and two loadings of different amounts of the *Sfi*I digested chromosomal DNA (lanes 2 and 3). Fragment assignment is according to ref. 13. The autoradiogram (lanes 4–6) shows the hybridization of fragment F to the radioactively labelled *swi7* probe (lanes 5 and 6). The top band of hybridization represents the DNA that remained in the loading well. The filter was subsequently probed with λ DNA to show the marker bands (lane 4). Conditions for radioactive labelling of the probes, hybridization and washing were as described in the legend to Fig. 2.

chromosome I (ref. 13). These results establish the mapping of *pola* on chromosome I.

Switching occurs spontaneously with high efficiency and with a regulated pattern in mitotic pedigrees. Only one among four grand-daughters of a cell switches in 80–90% of cell pedigrees¹⁴ and the sister of the recently switched cell is switching-competent^{5,15}. Earlier work has shown that the competence for switching of two *mat1* loci in diploid cells segregated independently for each chromosome¹⁶ and is governed by the inheritance of a specific DNA strand from the parental cell^{4,5}. It was postulated that the potential to switch is manifested by the ability to make the DSB in one specific chromatid, presumably during replication, and to inherit it in one of the daughter cells. It is not known when the break is made and when it is healed by switching in the cell cycle. Although it is not a direct demonstration, the requirement for DNA polymerase- α for DSB generation is the first evidence that the break may be made during replication.

DNA polymerase- α mainly carries out lagging-strand synthesis but is also required for initiating leading strand synthesis during DNA replication¹⁷. It may play a role in making the DSB by directly associating with and stimulating an unknown site-specific endonuclease or by inhibiting some factor involved in healing the break. Alternatively, DNA polymerase- α may act by indirect mechanisms. It is possible that a unique feature of *mat1* DNA replication plays a role in generating the DSB, for

example by initiating replication from a specific origin of replication. Aberrant breaks have been noted near the DSB site in *swi7-1* mutants, indicating cutting with reduced fidelity¹⁸. One possibility is that DNA polymerase- α may normally pause at the cut site to allow sufficient time for cleavage by the endonuclease; the *swi7-1* mutant may be defective in pausing, resulting in reduced fidelity. DNA polymerase- α might also be involved in repairing and stabilizing the ends of the DSB once it is formed. The mutant enzyme may not initiate replication efficiently at the proper origin; such a delay in replication around *mat1* may prevent action by other factors that cause the DSB only at a specific point in the cell cycle. Among the genes that play a role in DSB generation, *swi7* is the first whose biochemical activity has been identified. Further studies are needed to define whether *swi7* functions directly or indirectly.

DNA replication has been shown to be involved in stably turning genes on or off in all cells of several systems. Examples include repression of the silent mating-type loci in budding yeast^{19,20}, transcription of late genes in bacteriophage T4 and adenovirus²¹, and expression of gut markers in *Caenorhabditis elegans*²². We have previously proposed a model suggesting that DNA replication in fission yeast may impart developmental asymmetry for mating-type switching such a way that only one of the two daughters is affected^{4,5}. Results of this study provide some support for a role of DNA replication in dictating the programme of cellular differentiation. □

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Viral DNA and a viral peptide can act as cofactors of adenovirus virion proteinase activity

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HUMAN adenovirus (Ad2), like many other viruses¹, contains a virion-associated proteinase essential for the synthesis of infectious virus particles²⁻⁴. We observed proteinase activity in wild-type virus but not in the ts-1 virus², which contains a mutation in the Ad2 L3 endoprotease gene⁵ that confers temperature-sensitive processing of virion precursor proteins. Unexpectedly, we did not observe proteinase activity with purified recombinant^{6,7} endoprotease protein (*M_r* 23 K). Purified recombinant endoprotease protein, however, complemented the mutation in ts-1 virions, restoring proteinase activity when mixed together. This implied that cofactors may be required. Here we reconstitute proteinase activity *in vitro* with three purified viral components: (1) the recombinant endoprotease protein; (2) an 11-amino-acid peptide that originates from the carboxy terminus of pVI, the precursor to virion component VI; and (3) adenovirus DNA. The use of DNA for a proteinase activity is unprecedented.

Proteinase activity of disrupted wild-type virus was readily detected (Fig. 1a, bar 1) with an assay we developed (W.J.M. *et al.*, manuscript in preparation) using a new fluorogenic substrate (Leu-Arg-Gly-Gly-NH)₂-rhodamine which contains an amino acid sequence corresponding to a consensus sequence^{6,8,9} on the amino-terminal side of cleavage sites in Ad2 virion precursor proteins. As anticipated, no proteinase activity was detected with disrupted ts-1 virus assayed under identical conditions (Fig. 1a, bar 2). Unexpectedly, no proteinase activity was detected in an assay of purified recombinant endoprotease (rEP)

protein (Fig. 1a, bar 3). But full proteinase activity was obtained on incubation of disrupted ts-1 virus with the rEP protein (Fig. 1a, bar 4). This implied that cofactors required for proteinase activity may be present in Ad2 virions.

One cofactor is Ad2 DNA. When disrupted ts-1 virus was incubated with DNase and the DNase inactivated, 94% of the proteinase activity was lost (Fig. 1b, bar 1). Addition of Ad2 DNA resulted in a 12-fold increase in proteinase activity. In a control experiment with inactivated DNase, more than 92% of the proteinase activity remained (Fig. 1b, bar 2) relative to activity in the absence of treatment with any DNase (Fig. 1b, bar 3). If the only cofactor for proteinase activity were Ad2 DNA, then incubation of the rEP protein with wild-type Ad2 DNA would result in reconstitution of proteinase activity. But no reconstitution was observed (Fig. 1b, bar 4). This implied that other cofactors required for proteinase activity may be present in Ad2 virions.

The second cofactor is a peptide. When disrupted ts-1 virus was incubated with plasmin for 4 min and the plasmin inactivated before the addition of the rEP protein, no proteinase activity was observed (Fig. 1c, bar 2). Almost full proteinase activity (Fig. 1c, bar 1) was obtained in an identical experiment, except the plasmin had been inactivated before incubation with ts-1 virus (Fig. 1c, bar 3). Thus this second cofactor is a plasmin-sensitive virion protein. The second cofactor was purified from wild-type Ad2 virus and identified as Gly-Val-Gln-Ser-Leu-Lys-Arg-Arg-Arg-Cys-Phe (W.F.M., W.J.M., D.L.T. and C.W.A., manuscript in preparation). This sequence is present at the carboxy terminus of pVI, the 250-amino-acid precursor protein to virion component VI (ref. 10). A proteinase consensus cleavage sequence Ile-Val-Gly-Leu begins at residue 236; cleavage at the Leu-Gly bond would liberate the 11-amino-acid second cofactor.

Proteinase activity was reconstituted *in vitro* with the three purified components: the rEP protein, a chemically synthesized 11-amino-acid second cofactor (pVI-c peptide) and Ad2 DNA. Proteinase activity was stimulated by the presence of pVI-c peptide (Fig. 2a). In the presence of a constant amount of rEP

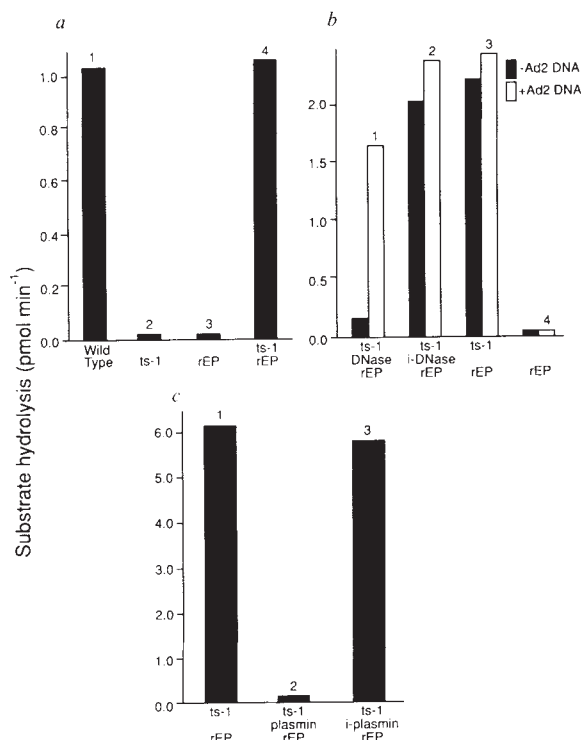


FIG. 1 Cofactors of Ad2 proteinase activity. *a*, Assay of (1) wild-type virus, (2) ts-1 virus, (3) rEP protein and (4) ts-1 virus plus rEP protein. Reactions were done in 400 μ l assay buffer which contained 50 mM Tris-HCl (pH 7.4), 25 mM NaCl, 10 mM octylglucoside, 1 mM dithiothreitol and 5 μ M (Leu-Arg-Gly-Gly-NH)₂-rhodamine. When present, the number of disrupted virus particles was 1×10^{10} and the concentration of rEP protein was 4.4 nM. After 30 min at 37 $^{\circ}$ C, a 360 μ l aliquot of the reaction was added to 340 μ l of assay buffer minus octylglucoside, and the increase in fluorescence was measured¹¹. *b*, Effect of DNase and Ad2 DNA on the cofactor activities in ts-1 virus. In (1), 2×10^{10} disrupted ts-1 virions were incubated in 20 μ l containing 0.1 M sodium acetate (pH 5.0), 5 mM MgCl₂, and 55 μ g ml⁻¹ DNase I for 4 min at 25 $^{\circ}$ C. Then EDTA to 23 mM and dithiothreitol to 2.3 mM were added and the reactions heated at 56 $^{\circ}$ C for 1 min to inactivate the DNase. The reactions were diluted by the addition of 380 μ l of assay buffer containing substrate, 18 nM rEP protein and either 2×10^{10} molecules of Ad2 DNA (open bars) or no DNA (solid bars) and assayed. The assays in (2) and (3) were identical to those in (1), except that in (2) the DNase was inactivated before addition (i-DNase), and in (3) no DNase was present. In (4), rEP protein plus 1×10^{10} molecules of Ad2 DNA were preincubated for 10 min at 37 $^{\circ}$ C before adding the substrate. *c*, Effect of plasmin on the cofactor activities in ts-1 virus. In (1), 4×10^{10} ts-1 virions and 18 nM rEP protein were preincubated for 10 min at 37 $^{\circ}$ C before adding substrate. In (2), ts-1 virus was incubated in 20 μ l of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, and 33 nM plasmin for 4 min at 37 $^{\circ}$ C. Dithiothreitol was added to 2 mM and the solution heated at 56 $^{\circ}$ C for 1 min, to inactivate the plasmin. Then rEP protein and substrate were added. The assay in (3) was identical to that in (2), except the plasmin was inactivated (i-plasmin) by heating to 56 $^{\circ}$ C in the presence of 2 mM dithiothreitol.

METHODS. The preparation of wild-type and mutant H2ts-1 viruses and of viral DNA have been described⁵. Virus twice banded in CsCl was suspended in 10 mM Tris-HCl (pH 6.8) containing 20% (v/v) glycerol and, after three 10 s bursts of sonication, was stored at -20 $^{\circ}$ C. Disrupted virus prepared this way was used as the source of virion proteinase activity.

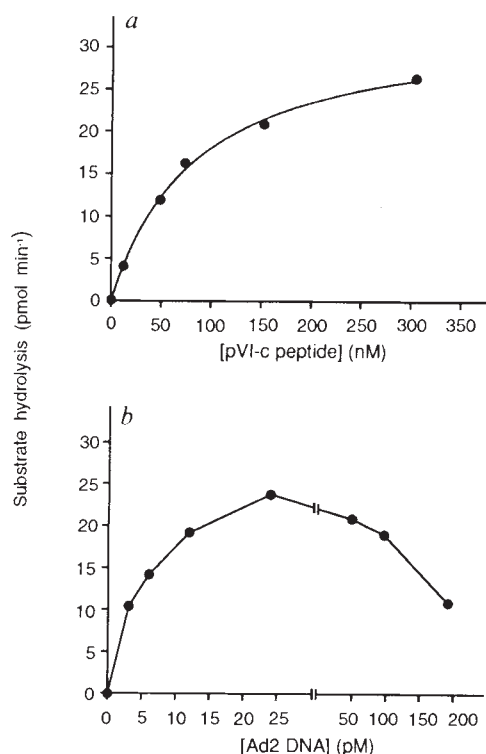


FIG. 2 Reconstitution of proteinase activity *in vitro* with purified components: titration with (a) pVI-c peptide and (b) Ad2 DNA. *a*, Assays in 1 ml contained 1.2 nM rEP protein, 11.9 pM Ad2 DNA and the indicated concentrations of pVI-c peptide. The rate of substrate hydrolysis is the rate in the reaction minus the rate with the rEP protein and Ad2 DNA alone which was 0.068 pmol min⁻¹. *b*, Assays in 1 ml contained 1.2 nM rEP protein, 36 nM pVI-c peptide and the indicated concentrations of Ad2 DNA. The rate of substrate hydrolysis is the rate in the reaction minus the rate with the rEP protein and pVI-c alone which was 2.76 pmol min⁻¹. The concentration of (Leu-Arg-Gly-Gly-NH)₂-rhodamine was 5 μ M, and its rate of hydrolysis to Leu-Arg-Gly-Gly-NH-rhodamine was determined by measuring the increase in fluorescence every 3 min for 15 min. The pVI-C peptide was assembled in a peptide synthesizer.

protein and Ad2 DNA, the amount of proteinase activity was proportional to the amount of pVI-c peptide. At higher concentrations, saturation was approached. Proteinase activity was stimulated by the presence of Ad2 DNA (Fig. 2b). As the Ad2 DNA concentration was increased in the presence of a constant amount of rEP protein and pVI-c peptide, proteinase activity rose, approached a plateau and, at high DNA concentrations, began to decrease.

Is there sequence specificity in the requirement *in vitro* for Ad2 DNA or for the pVI-c peptide? To determine whether proteinase activity was dependent on specific nucleotide sequences, we substituted various polymers for Ad2 DNA (Table 1). No sequence-specific requirement was evident. Not only did T7 DNA substitute for Ad2 DNA, but also single-stranded DNAs, circular single- and double-stranded DNAs, and even transfer RNA. Rather, it appeared as if the requirement was for a polymer

TABLE 1 Activation of Ad2 proteinase activity by negatively charged polymers

Effector* (concentration)	Rate† (pmol substrate min ⁻¹)	Stimulation‡ (fold)
None	2.8	1.0
Double-stranded DNA		
Linear		
Ad2 (5.9 pM)	25.6	9.3
T7 (2.5 pM)	26.1	9.5
Circular		
SV40 (27.9 pM)	24.4	8.9
ϕ X174 RF (18 pM)	23.3	8.5
Single-stranded DNA		
Linear		
M13 (+strand) (61 pM)	27.0	9.8
Circular		
ϕ X174 (+strand) (107 pM)	26.5	9.6
Four deoxyribonucleoside monophosphates (112 nM)	2.6	<1.0
RNA		
Yeast tRNA (10.2 nM)	19.8	7.2
Protein		
Polyglutamic acid (2.4 nM)	24.5	8.9
Polyaspartic acid (12 nM)	13.7	5.0
Polylysine (33 nM)	<1.0	<1.0
Amino acids		
Glutamic acid (780 nM)	2.3	<1.0
Aspartic acid (1.38 μ M)	2.1	<1.0
Heparin (33 nM)	12.9	4.7

* The concentrations of effectors in units of moles of molecules per litre that gave maximal stimulation of proteinase activity.

† All assays contained 8.3 nM rEP protein, 98 nM pVI-c peptide, and 1 μ M (Leu-Arg-Gly-Gly-NH)₂-rhodamine. The rate of substrate hydrolysis by the rEP protein alone was less than 0.014 pmol substrate min⁻¹; in the presence of 5.9 pmol Ad2 DNA, the rate of hydrolysis was 0.135 pmol substrate min⁻¹.

‡ The rate of hydrolysis of substrate in the presence of effector divided by the rate in its absence.

with high negative charge density. Polyglutamic acid, polyaspartic acid and heparin substituted for Ad2 DNA *in vitro* but not the four deoxyribonucleoside monophosphates, glutamic acid, aspartic acid or polylysine. The requirement for the 11-amino-acid second cofactor from the carboxy terminus of pVI seemed to be specific. A peptide similar in character, Cys-Gly-Tyr-Gly-Pro-Lys-Lys-Lys-Arg-Lys-Val-Gly-Gly, did not substitute for the pVI-c peptide (data not shown).

The viral DNA and pVI-c peptide may be required for proteinase activity *in vivo* to ensure that virion precursor proteins are processed only after virion assembly; otherwise, they may not be able to convene into a virus particle. Perhaps the viral DNA serves as a scaffold for the assembly of proteinase complexes adjacent to the >3,000 processing sites that must be cleaved to produce an infectious virus particle. Alternatively, the viral DNA could serve as a guide wire on which the proteinase complex moves as it cleaves precursor proteins.

The use of DNA as cofactor for a proteinase activity is unprecedented. The viral DNA is most probably required for proteinase activity in Ad2 virions, because proteinase activity with disrupted ts-1 virions is lost on treatment with DNase and is restored on addition of Ad2 DNA. □

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Unusual clustering of carboxyl side chains in the core of iron-free ribonucleotide reductase

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THE principal driving forces of protein folding are the burial of hydrophobic residues in the interior of proteins and the exposure of charged residues at the surface¹. Charged residues are only occasionally found in the interior, where they form hydrogen bonds to oppositely charged residues or main-chain atoms². Ribonucleotide reductase, a key enzyme in DNA synthesis, catalyses the *de novo* production of deoxyribonucleotide precursors. It is composed of two different dimeric proteins R1 and R2 (refs 3–5). R2 subunits contain buried iron-centres with each centre formed by two ferric ions coordinated by four carboxylates and two histidine ligands⁶. Iron-free R2, apoR2, is a precursor of active R2 and folds into a stable protein which is transformed into active R2 by ferrous ions and molecular oxygen. Here we show that the iron-free protein does not undergo any major structural changes compared with the iron-containing R2. The effect of this is a clustering of four carboxyl side chains in the interior of the subunit, in contrast to the normal distribution of charged residues in proteins.

ApoR2 was obtained by treating native protein with strong chelators under slightly denaturing conditions. During purification and crystallization this protein shows stability similar to that of the iron-containing form. ApoR2 can also be directly obtained from overproduction of the protein in bacteria under limited-iron conditions. ApoR2 and the active R2 protein can be crystallized under similar conditions at pH 6.0 (ref. 7). Diffraction data on apoR2 crystals were collected to 2.5 Å and the structure was refined to a crystallographic *R*-value of 18.7% (Table 1; Fig. 1). A comparison with the iron-containing protein shows no large global differences (Fig. 2). The observed differences are local and mainly restricted to the former metal sites and their environment.

In iron-containing R2 the positive charge of +6 from the two ferric ions is compensated by the two negative charges of the bridging O²⁻ and by the liganding Asp 84, Glu 115, Glu 204 and Glu 238 (ref. 6). In apoR2 the charges of the four acidic residues forming the metal site can be partly compensated for by protonation of the two former ligands His 118 and His 241. If all four carboxyl groups are deprotonated, the net charge at the former metal site inside the protein should be -2, but there is an additional buried carboxyl acid, Asp 237 hydrogen-bonded to His 118, so the buried net charge in this region of the protein

FIG. 1 An 'omit' $|F_o| - |F_c|$ map²³ of the metal coordination site in iron-free protein R2. The residues coordinating the iron ions in iron-containing R2 (Asp 84, Glu 115, His 118, Glu 204, Glu 238 and His 241) were removed from the structure, and 40 cycles of refinement were performed to relax the structure. The $|F_o| - |F_c|$ map, contoured at 3 σ , shows no density to indicate binding of other ions or water molecules in this region of the structure.

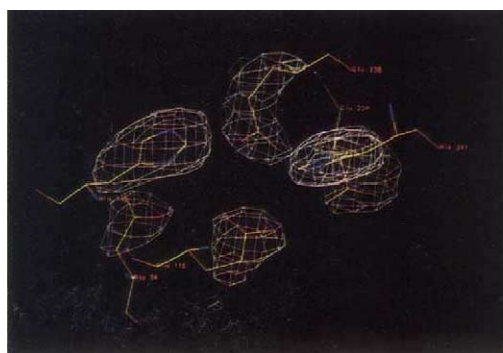


TABLE 1 Data collection and refinement

Spacegroup	$P2_12_12_1$, $a = 74.5$ Å, $b = 86.5$ Å, $c = 115.7$ Å
Statistics of data collection	
Resolution (Å)	60–2.5
N_{obs}	60, 248
N_{unique}	19, 365
R_{merge}	* 8.5%
Completeness	77%
Final refinement parameters	
No. of non-hydrogen atoms	5,877
No. of water molecules	293
Resolution (Å)	8.0–2.5
<i>R</i> -factor† (all reflections)	0.187
R.m.s. deviations from ideal geometry of the final model	
Bond distances	0.016 Å
Bond angles	3.2°
Dihedral angles	22.5°

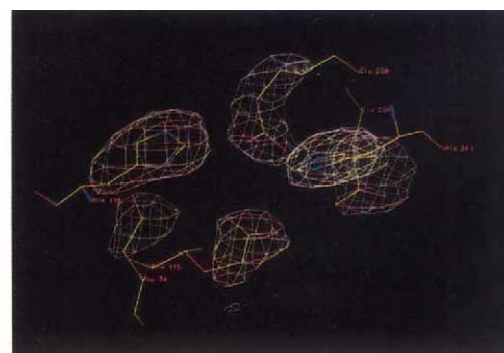
Structure determination: ApoR2 protein was prepared from iron-containing R2 by dialysis at 4 °C against a freshly prepared solution containing 50 mM lithium 8-hydroxyquinoline-5-sulphonate, 1 M imidazole-HCl, pH 7.0, and 30 mM NH₂OH-HCl (ref. 21). Crystals from apoR2 were made using the hanging-drop method. A 5-μl drop of protein solution (20 mg ml⁻¹) was mixed with 5 μl of buffered precipitation mixture. Crystals were grown from 20% polyethylene glycol (PEG 4000; Merck), 0.2 M NaCl in 0.05 M MES buffer (Sigma) at pH 6.0 in small hanging-drop chambers. The crystals used in this investigation were initiated by microseeding with normal iron-containing crystals using a standard dilution protocol, but identical crystals of similar quality can be obtained without seeding. Crystals of the iron-depleted apo-protein are almost isomorphous to native crystals⁶. A data set to 2.5 Å was collected on a Nicolet multiwire area detector mounted on a Rigaku rotating anode and evaluated using the Buddha program²². The CCP4 program suite (Daresbury, UK) was used for crystallographic computing. Data were first scaled and merged using Agrovata and Rotavata. The apoR2 reflections had isomorphous differences of 13% when compared to the native data set (calculated on *F*-values).

The refined R2 structure (P.N. and H.E. submitted) was used as a start model for simulated annealing refinement using the program X-plor (ref. 23); the initial *R*-factor for 6.5–2.8 Å reflections was 0.28. This was followed by positional and *B*-value refinement through energy minimization. Model-building during refinement was done using Frodo²⁴ and O (ref. 25). Structure comparisons were made using Program O. Coordinates of apoR2 will be deposited at the Brookhaven Data Bank.

$$* (\sum |I - \langle I \rangle| / \sum \langle I \rangle)$$

$$\dagger R\text{-factor } (\sum |F_{\text{obs}} - F_{\text{calc}}| / \sum F_{\text{obs}})$$

would be -3. However, the distances between Asp 84, Glu 115, Glu 204 and Glu 238 are short, indicating that some of them might be uncharged (Fig. 3a). Glutamic acid at position 115 and Glu 238 are central in this group and form hydrogen bonds to each other and to His 241. Glu 115 also forms hydrogen bonds to His 118 and to Asp 84, and Glu 238 hydrogen-bonds to Glu 204. This cluster of side chains shows a pseudo-twofold symmetry⁶, and the protonation pattern of the side chains is consistent with this symmetry. There are a few examples in other structures of pairs of carboxyl acids within hydrogen bond



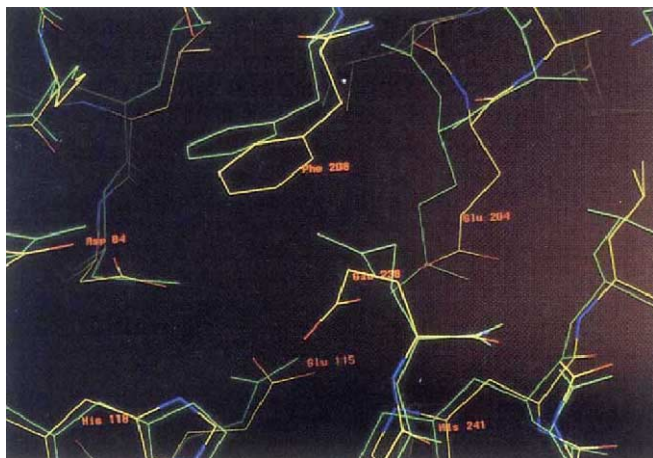


FIG. 2 Superposition of the refined structures of native R2 (green) and apoR2 (multicolours) using the Program O (ref. 25). The r.m.s. difference between C α atoms is 0.37 Å. All metal ligand side chains rearrange, especially Glu 238, which is about 2 Å closer to the iron-centre position in apoR2 than in the ferric R2 structure. Glu 204 is about 1 Å further away from the metal position in apoR2. Of non-liganding residues, Phe 208 at the proposed oxygen-binding site changes its conformation because the α E helix relaxes when Glu 204 does not bind a metal.

distance of each other² but no other example of a cluster of four carboxyl groups as in R2. In the aspartyl proteinases, where two charged residues of the same type are close to each other at the active sites, the pK_a of the residues is perturbed and one of the residues is protonated⁸.

All carboxyl side chains of the metal-binding site of apoR2 change their conformation somewhat and fill the hole created by the removal of the iron ions (Fig. 2). The dihedral angle of the side chain of Glu 238 changes rotamers for both χ_1 and χ_2 . Particularly significant changes are found for residues located on the long E helix (Glu 204 and Phe 208). The distortion found on this helix, due to the presence of one extra residue in a helix turn⁶, is also present in apoR2. This distortion may be important in providing the flexibility needed for binding of the binuclear iron cofactor. Most water molecules found in the iron-containing structure are also visible in the apoR2 maps. Some of the buried water molecules close to the iron centre are not present in the apoR2 structure, such as the two iron-bound water molecules. One water molecule hydrogen-bonded to Glu 204 is lost because of steric hindrance after rearrangement of this side chain.

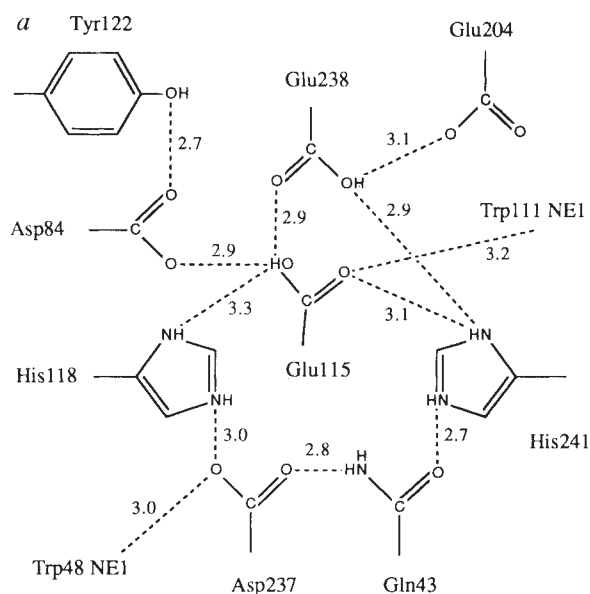


FIG. 3 *a*, A plausible hydrogen-bond system of the four carboxyl groups and the protonated histidines at the former iron-centre. Distances between hydrogen-bonding acceptor and donor atoms are given in Å. Five carboxyl groups in this cluster are buried inside the protein. The positions of the protons in the hydrogen-bond system are not unambiguous and may have other positions. There are no main-chain hydrogen bonds to the metal ligand

The three-dimensional structure of other metal-depleted metalloenzymes that show only small structural changes when the active site metal atom is removed have been reported^{9–11}. Removal of metals in concanavalin A results in notable conformational changes in the metal-binding region^{12,13}. Removal of calcium from proteinase K gives rise to small but long-range structural changes affecting the active site, which is 17 Å away from the metal site¹⁴. In all of these proteins, however, the metal is bound at or close to the surface.

In the structure of lactoferrin, the iron ion is buried inside the protein. When iron is released the protein goes through a large conformational change involving domain rotation and exposure of the empty metal-binding site to the solvent¹⁵. In the R2 protein the iron centre is located about 10 Å from the protein surface. Thus, one might expect a similar kind of opening of the iron-free structure to expose charged ligands and to allow entrance of ferrous ions. However, no large differences are found between the structures of apoR2 and iron-containing R2. A reason for this may be that R2 does not contain separate domains as lactoferrin but instead has a compact helix bundle structure.

The metal centre of R2 can be reconstituted in crystals of apoR2 without disturbance of the crystal lattice (ref. 16, and P.N., unpublished results), demonstrating that the metal centre can be formed without major conformational changes. If the



side chains. The net charge of the residues in the cluster in the figure is -1, the same as for ferric R2 (ref. 6). *b*, A hydrophilic core between four central helices (yellow ribbon) in the R2 subunit after the removal of iron from ribonucleotide reductase. The C α chain of the apoR2 subunit is shown in blue. The amino-acid side chains shown are ligands at the metal centre in the native protein.

preservation of the protein structure in metal-free forms is a preferred feature in biological systems, it imposes constraints on the evolution of these proteins and may explain why evolution seems to have disfavoured highly charged metal clusters and why the more hydrophobic porphyrin systems dominate in higher organisms^{17,18}.

The observed structure of apoR2 raises the question of how a polypeptide can fold into a globular protein with an interior of several carboxyl residues (Fig. 3*b*), a situation that may be analogous to carboxyl group clustering in, for example, some ion channels¹⁹. The folding of a protein is a highly cooperative process; in the case of R2 the energy cost of the clustering of carboxyl side chains in the interior of the protein²⁰ must have been accounted for by other means. Hydrogen-bonding to polar side chains in the vicinity of the carboxyl residues partly reduces the effect of the charges (Fig. 3*a*). Most importantly, the folded state of the subunit is stabilized by extensive van der Waals interactions and hydrogen bonds between the unusually long helices of R2. These interactions must then stabilize the structure sufficiently for the energetically unfavourable clustering of internal carboxyl residues to be tolerated. □

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ERRATUM

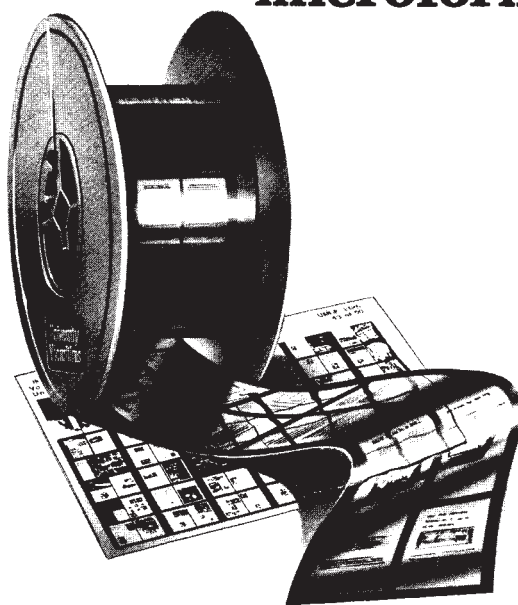
Phosphorylation of the *S. cerevisiae* Cdc25 in response to glucose results in its dissociation from Ras

Eitan Gross, Doron Goldberg & Alexander Levitzki

Nature **360**, 762-765 (1992)

THE sentence starting on line 29 of this letter should read "After 1 min the cAMP levels decay to an intermediate level and, in parallel, Cdc25 reaches an intermediate state of phosphorylation that is higher than the state of phosphorylation of Cdc25 in glucose-starved cells (Fig. 1*b*, lanes 5 and 1, respectively)."

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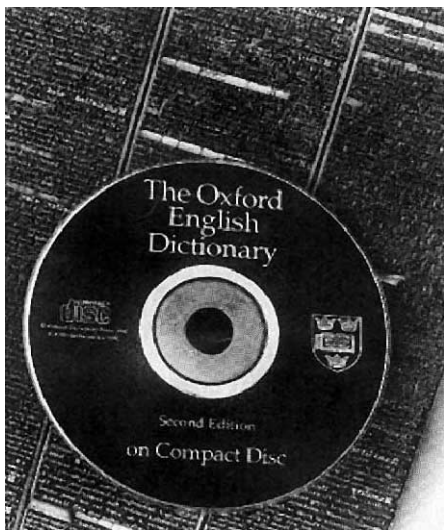
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Pick of the week

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Oxford English dictionary now on CD-ROM.

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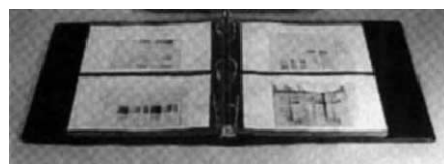
Researchers working in the pharmaceutical, agrochemical and fine chemical industries who regularly use protecting groups during the synthesis of organic molecules may be interested in the new **protecting groups database** from Synopsys, which provides thorough and systematic coverage of protection and deprotection reactions abstracted from the scientific literature (*Reader Service No. 102*). The database emphasizes reaction conditions and stability data — information that a chemist needs to make the right choice of protecting group to avoid costly experimentation. Synopsys says that indexing and cross-referencing of data from a wide variety of sources ensures that searching the database for relevant information about a protecting group is quick and easy. Using the facilities of REACCS, ORAC or ISIS, a chemist can search the database using the standard chemical and data search options provided. For example, a reaction substructure search could be used to find all methods in the database for protecting or deprotecting a particular functional group. Using keyword searches, a user can focus on protecting groups that are stable under the conditions to be used in subsequent reactions, or on ways of adding or removing a protecting group avoiding certain reaction conditions. The database covering the literature to the end of 1992 is being released in five increments of approximately 5,000 reactions, the first of which was released last month.

The synthesis of long GC-clamps used for analysis by denaturant gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE) can be tedious and costly. Moreover, GC-clamps may not be stable enough to allow some regions to be analysed. Appligene has introduced ChemiClamp **psoralene-linked oligonucleotides** to overcome some of these difficulties. Photoactivatable ChemiClamp stabilizes the end of the DNA fragment by

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Odds and ends

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Immunology update

C-C Biotech is offering Gerbu **adjuvant**, a powerful immunostimulator that is based on extensive testing using the glycopeptide GMDP (*N*-Acetylglucosaminyl-B(1-4)-*N*-acetylmuramyl-L-alanyl-D-isoglutamine) (*Reader Service No. 106*). The company says that the adjuvant promotes a strong immune response with a wide range of animals. It can be administered in an aqueous phase and, therefore, should possess little or no harmful side effects.

Anti-Fas monoclonal antibody from Kamiya Biomedical should be of interest to researchers studying apoptotic cell death (*Reader Service No. 107*). The monoclonal is a highly purified IgM mouse monoclonal antibody, which induces programmed cell death or apoptosis only on human cells that express the Fas antigen. The Fas antigen is a membrane-associated polypeptide that mediates apoptosis and is similar in structure to the tumour necrosis factor (TNF)/nerve growth factor receptor superfamily. It is found on various cells, including lymphocytes and various malignant human lymphoid cell lines. The monoclonal is prepared by immunizing a Balb/c mouse with human diploid fibroblast cell line/FS-7. Kamiya Biomedical says that this antibody does not cross-react with mouse Fas antigen, nor with the TNF receptor. It can be used for immunoblot or flow cytometry studies and should be of interest to researchers in the fields of immunology, embryology, AIDS and cancer.

DNA details

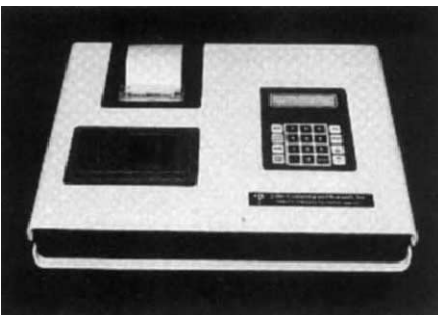
American Type Culture Collection, in collaboration with Maynard Olson's laboratory (University of Washington, Seattle, Washington), is distributing a **kit for mapping *Saccharomyces cerevisiae* sequences** by using the prime set of overlapping mapped genomic clones from *S. cerevisiae* AB972 (*Proc. natn. Acad. Sci. U.S.A.* **83**, 7826-7830, 1986; *Genetics* **127**, 681-698, 1991) (*Reader Service No. 108*). The US\$250 kit includes two hybridization membranes containing DNA from over 1170 clones covering over 95 per cent of the *Saccharomyces* genome, two transparent templates for localizing clone positions on the membrane, and one tube of positive control DNA. ASCII files containing data on the clone set and the clone/membrane position are provided on a floppy disk for use on either PC-DOS or Macintosh computers.

Life Technologies now offers Spectrum-Green **WCP DNA probes**, the newest additions to the company's line of *in situ* hybridization systems for identification of human chromosomes (*Reader Service No. 109*). This Gibco BRL whole chromosome painting system labels the entire target chro-

mosome with a fluorescent green dye that can visually be seen with the fluorescence-microscope filter sets already found in most laboratories. The WCP DNA probe in each whole chromosome painting system includes DNA sequences that are complementary to sequences found on the target chromosome. These probes are labelled directly with detectable fluorophores, eliminating the need for indirect detection methods required by other systems, the company says. For studying translocations, SpectrumGreen probes can be used together with SpectrumOrange probes for dual labelling on the same slide.

Means of measurement

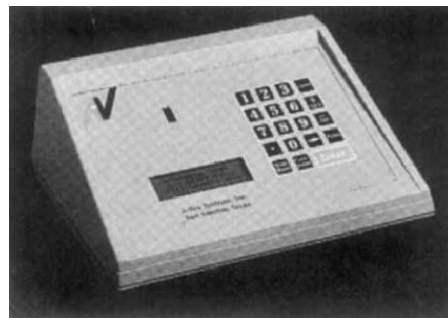
Fluorescence polarization can be a powerful technology that has many applications in biomedical research. It has been utilized in the detection and measurement of antigens, antibodies, DNA/RNA, enzymes, and in studies on living cells and their membranes. Jolley Consulting and Research says, however, that the instrumentation that is available at present can be costly, slow and of low precision and sensitivity. The company has introduced a high-performance instrument, the FPM-1, which is dedicated to the **measurement of fluorescence polarization and fluorescence intensity**



Fluorescence polarization analyser.

(*Reader Service No. 110*). Utilizing disposable 12×75 -mm glass test tubes, the FPM-1 is designed to be rapid (approximately five seconds per measurement) and precise (a standard deviation of <0.001 polarization units). The company says that the instrument is accurate at 10^{-11} M fluorescein in the polarization mode and down to 10^{-12} M in the intensity mode, making it suitable for the measurement of very low concentrations of dsDNA using TOTO or YOYO (Molecular Probes, Inc., Eugene, Oregon). The FPM-1 comes equipped with fluorescein filters as standard; custom filters are available on request. Excitation and emission filters are easily interchanged by the user. Four static and four kinetic modes of measurement are provided. Protocols are designed by the user and stored in nonvolatile memory. Output is available by hard copy and via a RS232 port to an external computer.

The new AVOXimeter 1000 from A-VOX Systems is both an **oximeter** and a **haemoglobinometer**: it measures the



AVOXimeter 1000 — more than an oximeter.

oxyhaemoglobin saturation, total haemoglobin concentration and oxygen content of blood samples (*Reader Service No. 111*). The company says that it uses safe, disposable cuvettes, requires no maintenance, no reagents and no sample preparation. The operator injects 50 μ l of whole blood into a cuvette and inserts the cuvette into the instrument. In less than 10 s, the AVOXimeter 1000 shows the readings on the lighted display, stores the results in the memory, or sends them to a printer (which is optional). Carboxyhaemoglobin, methemoglobin, haemolysis, and diagnostic concentrations of indocyanine dye are said not to affect the accuracy of the measurements. Suitable for use on haemoglobin solutions in addition to whole blood from both humans and animals, the instrument also performs haemodynamic computations including Fick Principle cardiac output. Calibration and quality control are designed to be easy with the commercially available standards.

Under the microscope

Bio-Rad has launched a new, true colour, **portable video microscope** range, the Multiscope range, which enables the operator to inspect subjects that are often difficult to image using conventional microscopic or photomicrographic techniques (*Reader Service No. 112*). Multiscopes are configured with the latest in miniature CCD cameras, fibre-optic illumination and optical system housed in a small, hand-held unit that is designed to offer unparalleled freedom and flexibility over conventional microscopes.



Hand-held microscopes — see Bio-Rad.

Bio-Rad says that the dual-magnification system of the Multiscopes not only produces high-quality images with greatly enhanced depth of field but also eliminates tedious lens changes. High and low magnification adaptors are also available, providing a range of magnifications to suit all applications in industry, life sciences and teaching. Multiscopes are fully compatible with a wide range of video monitors, recording equipment, video printers and archiving systems enabling upgrade from standalone imaging applications through to advanced documentation.

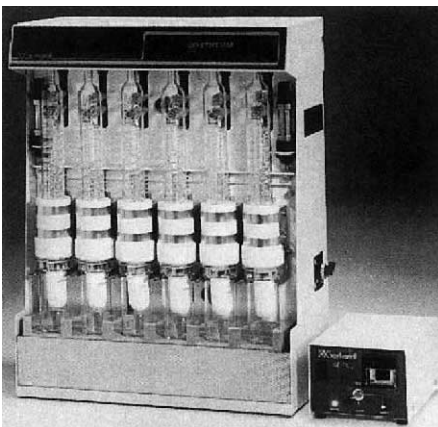
Send images anywhere in seconds with the ImageManager from Roche Image Analysis Systems (*Reader Service No. 113*). With it, the operator can **store, catalogue, sort and retrieve images**, as well as transmit them over ordinary telephone lines to any similarly equipped workstation. For example, the operator might choose patient name, identification number, disease type, origin, stain size or laboratory specialty as key words for the storage of images. The company says that the operator can in fact use up to 18 user-defined fields as key words so that the images can be accessed in practically any way the operator wishes. The ImageManager uses a Microsoft Windows graphical user interface.

New lines in labware

The new accessories for the Vibratome **fresh tissue sectioning systems** from Technical Products International are designed to increase the versatility of the instruments, making them more useful tools in the laboratory (*Reader Service No. 114*). The rotating stage assembly allows a specimen to be oriented to the blade after it has been affixed, with adhesive, to the specimen mount. At a 20° blade angle, the rotating stage assembly will adjust up to 15°, still allowing 10-mm clearance. The rotating stage assembly, rotating specimen mounts, V-block adapter, specimen tray, sapphire knife and glass knife adapter can all be used with either the Vibratome Series 1000 or the newer Series 2000 models. All Series 1000 models can be retrofitted to the Series 2000

status featuring bath refrigeration, automatic sectioning repetition and motorized digital control of section thickness.

The new generation of Soxtherm 2000 **automatic fat extraction systems** is now available from Gerhardt (*Reader Service No. 115*). The systems are designed to simplify the Soxhlet method, operating up to five-times faster than the traditional method.



Gerhardt's fat extraction systems.

The spark-secured electrical heating makes temperatures up to 300 °C possible (precision 1K) and can therefore be used for all kinds of samples and solvents. The extraction runs in four phases: boiling stage, solvent lowering phase, washing phase and solvent recovery phase, which are controlled by a pneumatically operated switch in version M and by microprocessor in version A. Up to 90 per cent of the solvent is collected in the built-in storage tank and can be reused. Six determinations can be run simultaneously.

ThermAdapt is a new **thermistor-based thermoregulator** from Biophysica Technologies that provides microprocessor temperature control from -10 to 50 °C with a precision of ± 0.1 °C for many laboratory applications (*Reader Service No. 116*). Its artificial intelligence and fuzzy logic control program adapts to the heat dissipation properties of the apparatus under control for fast equilibrium without overshoot. The company says that calibration by the operator is not necessary. A novel learning paradigm called OptiSet can be engaged to optimize further equilibration time and



ThermAdapt — thermoregulation for a wide variety of laboratory equipment.

stability for up to five different consistent types (channels) of study. The temperature is set and displayed continuously on an LED digital panel meter and the unit can also be interfaced, monitored and additionally custom-programmed by computer. Other features include ramp and soak programming, voltage output override, battery backed-up memory and self-diagnostics. The device can be used for the temperature control of waterbaths, microscope tissue chambers, brain-slice chambers and various culture instruments.

The new DSC-50Q **differential scanning calorimeter**, designed by Shimadzu and available from V. A. Howe, has quench cooling capabilities (500 °C min⁻¹), making it suitable for the characterization of the amorphous part of the polymer (*Reader Service No. 117*). Furthermore, it is possible to run a controlled cyclic temperature program, which is intended to reduce the influence of the former thermal history of the sample. The DSC-50Q can be used for all other kinds of studies that recommend a cyclic temperature programme or need to be run in sub-ambient temperatures.

The Finnigan MAT SOLA is a **modular mass spectrometer system** for multi-element analysis of liquids and solids (*Reader Service No. 118*). It is characterized by high-performance Turner ion optics with the choice of two plasma sources. Both inductively-coupled plasma mass spectrometry (ICP-MS) and glow discharge mass spectrometry (GD-MS) are resident in one instrument. These two ion sources have both become widely used for multielement analysis. They produce simple, clear atomic spectra, with less interference and equivalent or higher sensitivity than is achievable by either spark source or optical emission systems. The modular design of SOLA allows the ICP and GD sources to be interchanged. The ICP source is predominantly used for the analysis of liquids, with the sample solution introduced into the RF plasma region as very fine spray. The GD source is used to measure directly the elemental composition of solid samples without any acid digestion. The quadrupole mass analyser uses three stages of differential pumping to sample the ion population. Finnigan MAT says that careful design of the interface and ion transfer optics has produced an order of magnitude increase in sensitivity, and a reduction in photon and neutral background. Multitasking software on a 486 PC with extended memory directs instrument control and data handling. □

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